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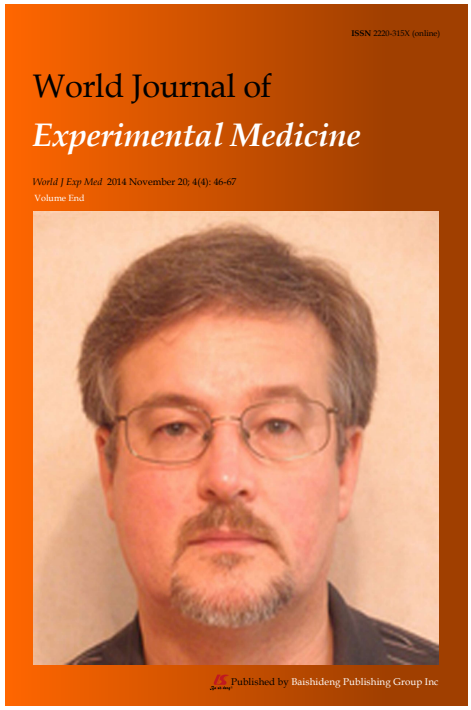

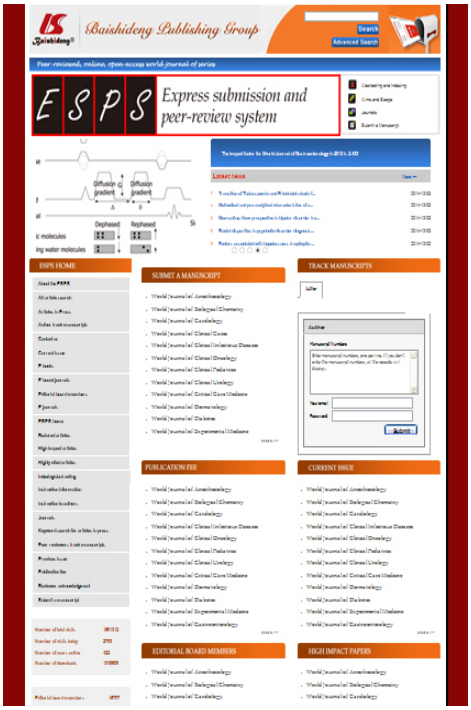

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EDITORIAL

High density lipoproteins and type 2 diabetes: Emerging concepts in their relationship

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Abstract

Patients with type 2 diabetes mellitus (T2DM) frequently exhibit macrovascular complications of atherosclerotic cardiovascular (CV) disease. High density lipoproteins (HDL) are protective against atherosclerosis. Low levels of HDL cholesterol (HDL-C) independently contribute to CV risk. Patients with T2DM not only exhibit low HDL-C, but also dysfunctional HDL. Furthermore, low concentration of HDL may increase the risk for the development of T2DM through a decreased β cell survival and secretory function. In this paper, we discuss emerging concepts in the relationship of T2DM with HDL.

Key words: Type 2 diabetes; High density lipoproteins; Insulin secretion; β cells; Paraoxonase-1

Core tip: Patients with type 2 diabetes mellitus (T2DM) not only exhibit low high density lipoprotein (HDL) cholesterol, but also dysfunctional HDL. Furthermore, low concentration of HDL may increase the risk for the development of T2DM through a decreased β cell survival and secretory function. In this paper, we discuss emerging concepts in the relationship of T2DM with HDL.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) affects approximately 12 million people in the United States[1]. Atherosclerotic cardiovascular (CV) disease accounts for about 70% of overall mortality in patients with T2DM[2,3]. Various factors, modifiable or not, promote atherosclerosis in these patients[1]. These include metabolic abnormalities, such as hyperglycemia, hyperinsulinemia, albuminuria and atherogenic dyslipidemia [low high density lipoprotein cholesterol (HDL-C) together with increased triglycerides (TG) levels, as well as raised cholesterol concentration of the small dense low density lipoprotein (sdLDL) particles][1,4-7].

Atherogenic dyslipidemia is characterized by the imbalance between pro-atherogenic apolipoprotein (apo)B-containing and anti-atherogenic apoA1-containing lipoprotein particles[8]. In this context, sdLDL particles predominate[9-13]. The small size of LDL particles has been recognized as a risk predictor of CV events[10,11,14].

Interestingly, the risk of coronary heart disease (CHD) associated with atherogenic dyslipidemia may exceed the risk from raised low density lipoprotein cholesterol (LDL-C) levels of 150-220 mg/dL[1,7]. Furthermore, even statin-treated patients with T2DM within LDL-C goals exhibit residual CV risk, which is partially associated with the presence of atherogenic dyslipidemia[15,16]. A *post hoc* analysis of the United Kingdom Prospective Diabetes Study (UKPDS) assessed the CV risk across quintiles of log(TG)/HDL-C in 585 men with T2DM[17]. The risk for CHD or cerebrovascular events was augmented at the highest compared with the lowest quintile (28% *vs* 52%,

respectively, $P = 0.001$)[17].

Except for the predominance of sdLDL particles, low HDL-C levels comprise an independent risk factor of CV events[18,19]. In the Framingham Heart Study, HDL-C was a more potent predictor of CHD than total cholesterol, LDL-C or TG[18]. It was suggested that for every 1 mg/dL decrease in HDL-C levels, the risk for CHD increases by 2% in men and 3% in women[20].

HDL is responsible for the process of reverse cholesterol transport from peripheral tissues, including arterial wall, to the liver[21]. Furthermore, HDL exhibits multiple anti-atherogenic actions[21,22]. These include anti-inflammatory, anti-oxidant and anti-thrombotic effects together with an HDL-associated restoration of endothelial function[21,22]. These actions are mediated at least in part by the enrichment of HDL with apoA1 or enzymes [*e.g.,* paraoxonase-1 (PON1) and HDL-associated lipoprotein-associated phospholipase A_2 (Lp-PLA_2)][21,23,24]. In this paper, we discuss the relationship between T2DM and HDL.

LOW LEVELS OF HDL-C IN T2DM

Patients with T2DM exhibit low HDL-C levels[12]. Among 7692 outpatients with T2DM, the prevalence of low HDL-C levels (< 40 and 50 mg/dL for men and women, respectively) was 49.5%[25]. Several mechanisms have been described to explain this abnormality mostly associated with the predominance of TG-rich lipoproteins[12,23,26]. Briefly, very low density lipoproteins (VLDL) are overproduced in insulin resistant states[12,23,26]. Furthermore, insulin resistance is associated with a defective clearance of TG-rich lipoproteins (*i.e.,* VLDL, chylomicrons and their remnants) *via* lipoprotein lipase (LPL)[23]. These lipoproteins exchange their core lipids with HDL through cholesterol ester transfer protein (CETP) resulting in TG-enriched HDL particles[27]. The activity of CETP is enhanced in insulin resistant states (*e.g.,* T2DM)[27]. The enrichment of HDL particles with TG decreases the stability and plasma residence time of these lipoproteins[23,28,29]. Namely, apoA1 is easily removed from circulating TG-rich HDL particles following lipolysis[23,28]. Furthermore, the lipolysis of these lipoproteins by hepatic lipase gives rise to small HDL particles, which are rapidly cleared[23,28]. Also, hypertriglyceridemic states are characterized by reduced availability of the lipolytic surface fragments derived from TG-rich lipoproteins. These components are necessary for the formation of HDL[23,28].

DYSFUNCTIONAL HDL IN T2DM

It was suggested that HDL is dysfunctional in T2DM. Experimental *in vivo* and *in vitro* studies showed that HDL-associated reverse cholesterol transport is impaired in T2DM[30-32]. Several mechanisms were suggested to mediate this abnormality. These include a reduced expression of the ATP-binding cassette (ABC) transporters. The members A1 and G1 of this family facilitate the efflux of cellular free cholesterol and phospholipid to assemble with apoA1 and form nascent HDL[33]. The gene expression and protein levels of ABC-A1 were reduced in T2DM in parallel with poor glycemic control[32]. This may increase risk for CHD[34]. Furthermore, insulin decreased the *in vitro* protein expression and activity of ABC-G1[35]. This finding suggests a role of hyperinsulinemia (*e.g.,* in T2DM) in defective HDL-mediated reverse cholesterol transport.

The oxidative modification of HDL (especially of apoA1) by glycated hemoglobin may be another mechanism explaining HDL dysfunctionality in T2DM[30,31]. This could be related to the presence of the haptoglobin Hp2 allele, which increases the oxidative modification of circulating lipoproteins[31]. Experimental data showed that HDL dysfunctionality in T2DM may be ameliorated by the use of antioxidants (*e.g.,* vitamin E) *in vivo*[36]. Furthermore, the anti-oxidant defense of HDL is decreased in T2DM. This could be associated with a reduced PON1 activity mediated by the glycation of this enzyme[37-39]. Of interest, postprandial glycemia and impaired catabolism of TG-rich lipoproteins was associated with decreased PON1 activity in T2DM[40-42]. Several polymorphisms of *PON1* gene favor the defective action of PON1 in T2DM[43]. Reduced PON1 activity was an independent predictor of CV events in patients with T2DM[44].

We have previously shown that patients with metabolic syndrome exhibit decreased activity of HDL-associated Lp-PLA_2 compared with age and sex-matched controls[45,46]. HDL-associated Lp-PLA_2 contributes significantly to the anti-inflammatory and anti-atherogenic potential of HDL[47,48]. Despite low activity of this enzyme in pre-diabetic insulin resistant states, data are insufficient for patients with T2DM.

PROTECTIVE ROLE OF HDL IN THE PATHOGENESIS OF T2DM

The gradual deterioration of pancreatic β cell function following persistent insulin resistance is the main pathophysiological event in T2DM[49,50]. At the time of T2DM diagnosis, the secretory function of β cells is declined by approximately 50% of normal[51]. It was suggested that lipoproteins may regulate glucose homeostasis by affecting both peripheral insulin resistance and pancreatic islet secretion[52]. For example, high circulating levels of free fatty acids impair insulin sensitivity[53-55]. The emerging concept is that atherogenic dyslipidemia may precede T2DM and favor its development by promoting the dysfunction and apoptosis of β cells[56]. In the UKPDS, the log(TG)/HDL-C ratio, as a surrogate of atherogenic dyslipidemia, was associated with decreased insulin sensitivity and impaired β cell function in 585 male patients with T2DM[17].

Low HDL-C levels independently predict the development of T2DM[57]. A recent observational study investigated the association of HDL-C and β cell function in 1087 subjects at risk of T2DM[58]. Low HDL-C levels

were independently associated with indices of β cell dysfunction in patients with impaired either fasting glucose or glucose tolerance[58].

Pancreatic β cells express receptors that participate in the binding and processing of plasma lipoproteins[53,59]. These include the LDL-receptor and the LDL-receptor related protein[60]. Both circulating and endogenous cholesterol of β cells can affect insulin secretion[60]. In this context, VLDL and LDL particles reduce insulin mRNA expression and proliferation, while inducing apoptosis of β cells[53]. Furthermore, cholesterol accumulation in pancreatic β cells may impair their secretory function[52,60,61]. In contrast, HDL exerts a protective role by improving β cell secretory function and antagonizing the apoptosis of these cells[53]. The lipid-free apoA1 and apoA2 or HDL increased insulin secretion by up to 5-fold *in vitro*[62]. Furthermore, the administration of reconstituted HDL in patients with T2DM improved the glycemic control by increasing β cell insulin secretory function[63].

The process of reverse cholesterol transport can help explain these benefits. Several experimental studies highlighted the protective role of ABC-A1 against T2DM[60]. In contrast, ABC-A1 knockout mice exhibited impaired glucose tolerance due to a decreased insulin secretion upon glucose stimulation[64]. This effect was not accompanied by any changes in insulin mRNA expression, suggesting that cholesterol accumulation in β cells interferes with insulin exocytosis[52,64]. Furthermore, human carriers of loss-of-function ABC-A1 mutations exhibited reduced not only HDL-C levels, but also insulin secretion[63,65]. On the other hand, rosiglitazone improved glucose tolerance by upregulating the expression of ABC-A1 gene[64].

Several *in vitro* studies suggested a beneficial role of HDL on the survival of β cells[53,56,66]. This benefit may be mediated by the anti-oxidant effects of HDL. For example, oxidized LDL (oxLDL) decreased insulin secretion at the transcriptional level and promoted apoptosis of β cells *in vitro*[66]. This was associated with an activation of the Jun N-terminal kinase pathway[66,67]. HDL reversed these actions of oxLDL[66]. To this extent, experimental studies showed that PON1 increases insulin secretion, thereby reducing the incidence of T2DM *in vivo*[68,69]. PON1 was also associated with increased survival of β cells[69]. Furthermore, not only PON1 but also HDL-associated Lp-PLA$_2$ inhibits the oxidation of LDL[70]. Lp-PLA$_2$ is produced in the arterial wall by macrophages[70]. It is associated with lipoproteins, primarily LDL and secondarily HDL, and degrades bioactive phospholipids[70]. Both PON1 and HDL-associated Lp-PLA$_2$ protected hypercholesterolemic mice from atherosclerosis[71,72]. OxLDL inhibit these enzymes[72]. Therefore, oxLDL and HDL are considered antagonists in the development of atherosclerotic vascular disease[72].

CONCLUSION

Interest is increasing on the protective role of HDL against atherosclerotic CV disease. CV risk is high even in patients with T2DM who exhibit LDL-C levels within normal range. Low HDL-C is an independent contributor of this residual risk. The increased concentration of circulating TG-rich lipoproteins mostly accounts for low HDL-C levels in patients with T2DM. Considerable evidence suggests that HDL is dysfunctional in T2DM. Indeed, decreased ABC-A1 and/or -G1 expression reduces biosynthesis of HDL in T2DM through reduced availability of cholesterol for loading to apoA1. This results in impaired reverse cholesterol transport. Furthermore, the oxidative modification of HDL (especially of apoA1) in T2DM impairs its functionality. This is in part associated with a reduced anti-oxidant defense of these lipoproteins *via* PON1. The emerging concept is that low HDL-C may be involved in the pathogenesis of T2DM. The abundance of circulating atherogenic particles together with the increased intracellular cholesterol concentration in β cells have been associated with impaired secretory function of pancreatic islets. HDL by removing cholesterol from these cells may increase insulin secretion. Furthermore, these lipoproteins increase the survival of β cells by mechanisms which are under investigation. The anti-oxidant actions of HDL *via* PON1 may play a key role in this benefit.

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and position-

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MINIREVIEWS

Animal models of *ex vivo* lung perfusion as a platform for transplantation research

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Abstract

Ex vivo lung perfusion (EVLP) is a powerful experimental model for isolated lung research. EVLP allows for the lungs to be manipulated and characterized in an external environment so that the effect of specific ventilation/perfusion variables can be studied independent of other confounding physiologic contributions. At the same time, EVLP allows for normal organ level function and real-time monitoring of pulmonary physiology and mechanics. As a result, this technique provides unique advantages over *in vivo* and *in vitro* models. Small and large animal models of EVLP have been developed and each of these models has their strengths and weaknesses. In this manuscript, we provide insight into the relative strengths of each model and describe how the development of advanced EVLP protocols is leading to a novel experimental platform that can be used to answer critical questions in pulmonary physiology and transplant medicine.

Key words: *Ex vivo* lung perfusion; Transplantation; Rat; Porcine; Small animal; Large animal; Model; *Ex vivo* lung perfusion

Core tip: *Ex vivo* lung perfusion allows for lungs to be assessed for their physiologic and functional parameters prior to transplant. As a tool for experimental research, the technology is an extremely powerful tool that enables isolated organ modification and evaluation. Utilizing small and large animal models have complementary approaches to addressing transplant related questions.

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INTRODUCTION

Overview of lung transplantation donor organ shortage

Lung transplants have become a viable option for patients with end stage lung disease. Unfortunately, only about 15% of donor lungs are deemed appropriate for transplant[1], and estimates show that about 50% of patients die while waiting for a lung transplant[2]. Addition-

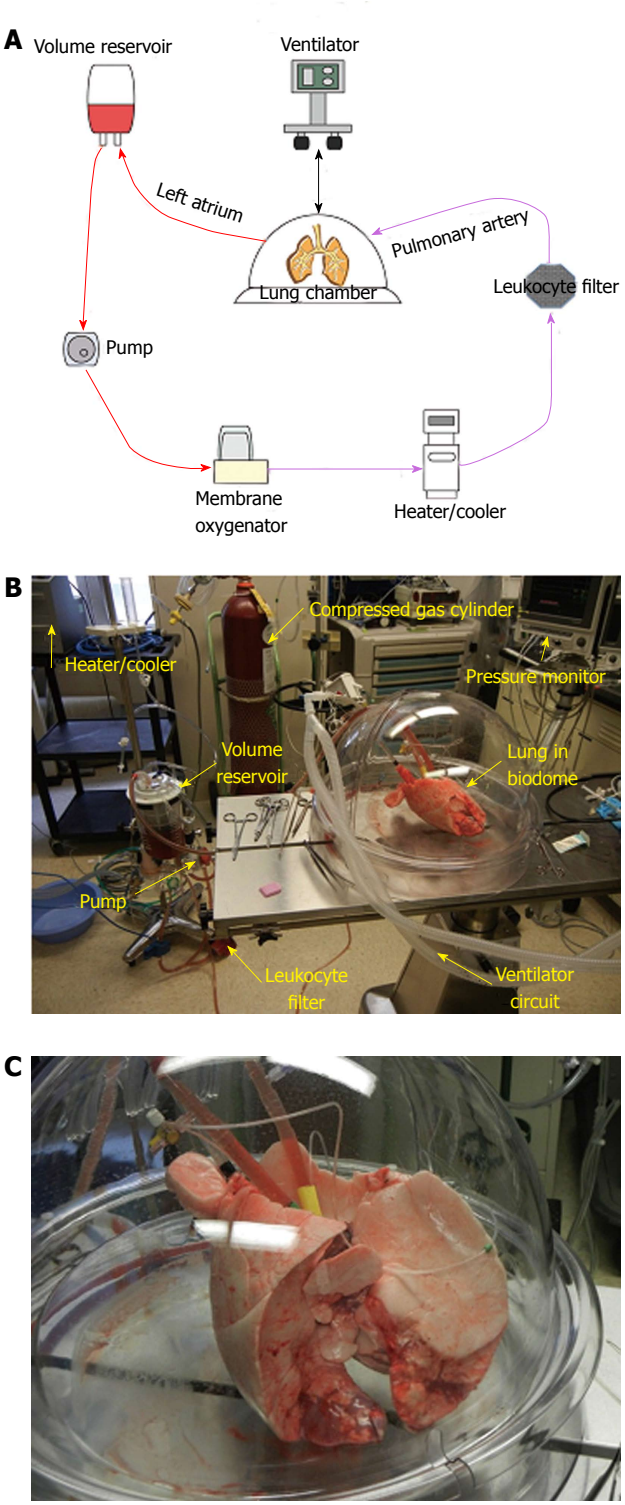

Figure 1 This diagram depicts a schematic of a large animal (porcine) *ex vivo* lung perfusion circuit (A), the portable stand for the perfusion pump (B), A close up picture of a porcine lung on an *ex vivo* lung perfusion circuit (C). The ventilator is used to expand the lungs with lung protective ventilation strategies. The volume reservoir contains the perfusates (either sanguineous or acelluar). Centrifugal pumps have the advantage of being able to have the afterload varied and have the circuit clamped easily, which is a challenge with roller pumps. The heater/cooler allows for exact temperature titration. The reservoir, centrifugal pump, membrane oxygenator, and leukocyte filter are all contained on this apparatus. The biodome which houses the large animal lung is visualized with the ventilator circuit attached to the endotracheal tube which directly cannulates the trachea. The inflow and outflow cannulas are at the superior aspect of the lung and the endotracheal tube on image top left.

ally, the average patient who receives a lung transplant waits 412 d for a suitable lung[3]. Because so many lungs do not meet transplantation requirements, the quality of available lungs must be increased in order to increase the amount of lungs available for transplant.

Ex vivo lung perfusion

Ex vivo lung perfusion (EVLP) (Figure 1) has the potential to increase the lung donor pool and allows for precise control of important variables including perfusate composition, temperature, tidal volume, positive end expiratory pressure (PEEP), fraction of inspired oxygen, and arterial pressure. EVLP can improve donor lungs that were originally thought to be in too poor a condition to be transplanted and can also be used to determine a lung's condition for donation[4,5]. EVLP also allows for the assessment of donor lungs without having to transplant them to another person.

Current clinical state of EVLP

Steen *et al*[4] first published their paper on the transplant of a lung that was perfused *ex vivo* in 2007. In 2011, Both Cypel *et al*[6] and Lindstedt *et al*[7] reported that initially rejected lungs that were perfused *ex vivo* performed similarly to lungs that were initially selected for transplant. In 2012, both Aigner *et al*[8] and Zych *et al*[9] reported that EVLP has the potential to improve the quality of donor lungs that otherwise would not be selected for transplant. In 2013, Wallinder *et al*[10] reported the EVLP is a safe method and allows lungs that would have been rejected to be used in transplants. The potential impact of EVLP to expand the available organ donor pool is profound. If the lung donor conversion rate is able to be increased from 17% to 30%, that small incremental increase in donor conversion would ostensibly double the number of transplants able to be performed worldwide annually. Evaluating the mechanisms of lung injury and progression would enable targeted therapies to intervene on these critical set points. EVLP provides an isolated platform where these mechanical traumas can be isolated and evaluated in a mechanistic fashion. Through a combination of lung protective ventilation, reducing airway edema, and targeted therapies we would anticipate that the increase conversion rate would be able to be met.

EVLP AS AN EXPERIMENTAL PLATFORM

Evaluation of organ function

While performing EVLP, multiple factors can be assessed in real-time to determine the viability of the lung. These include pulmonary arterial flow, pulmonary arterial pressure and pulmonary resistance, as well as dissolved oxygen concentration in the perfusate before and after passing through the pulmonary circulation. This change in dissolved oxygen corresponds to the oxygen production by the lung. The wet-to-dry ratio of a lung can also be assessed, giving an accurate depiction of how edematous the lung has become.

Table 1 Dependent and independent variables with *ex vivo* lung perfusion

Dependent variables (*i.e.*, what can be measured with *ex vivo* lung perfusion)	Independent variables (*i.e.*, what can be varied in an *ex vivo* lung perfusion)
Tracheal pressure	Tracheal pressure
End expiratory pressure	End expiratory pressure
End inspiratory pressure	End inspiratory pressure
Tidal volume	Tidal volume
Compliance	Respiratory rate
Respiratory rate	Pulmonary artery flow rate
Pulmonary artery flow rate	Pulmonary artery pressure
Pulmonary artery pressure	Left atrial outflow pressure
Left atrial outflow pressure	Perfusate
Pulmonary vascular resistance	Ischemic time
Lung weight	Temperature of perfusate
Wet to dry ratio	Temperature of organ
Pre-organ pO_2	Inspired gas concentration and components
Post-organ pO_2	
Oxygen production	
Perfusate pH	
Perfusate pCO_2	
Perfusate for molecular analysis	
Tissue for mRNA, protein, or histologic analysis	

Model of acute lung injury development

EVLP can also be used as a model of acute lung injury (ALI) development and ventilation induced lung injury (VILI). Currently, there has been only one clinical trial that has resulted in a significant decrease in patient mortality related to ALI and VILI[11]. Although multiple ventilation protocols have been researched, there is little information on how drug treatment might affect lung viability at various tidal volumes and positive-end expiratory pressures. Multiple models of ALI that are typically used for *in vivo* studies (*i.e.*, saline lavage/surfactant dysfunction, acid induced lung injury and LPS induced lung injury) can be easily and quickly implemented using EVLP (Table 1). In addition, precise regulation of tidal volume, PEEP and other ventilator parameters during EVLP allow for modeling the mechanically induced injury that occurs during VILI. Unlike *in vivo* models, EVLP models of ALI/VILI allow for the evaluation of how specific ventilator settings influence lung injury progression without the confounding effects of changes in other physiologic parameters (Table 1). These models can be assessed by measuring pro-inflammatory cytokine secretion and histological characterization of lung tissue. The lungs can also be treated with specific drugs delivered through the perfusate or trans-trachealy to determine if any drug combination results in a minimization of lung damage during ventilation.

Pathway to evaluate efficacy of experimental treatments

Perfusate: The selected perfusate should have osmotic and oncotic pressures similar to blood and must also provide an energy source for the cells. Clinically, the perfusate is used to evenly cool the organ tissue and to remove blood, thereby preventing cell injury. It is important to note that the perfusate and all of its components have direct contact with the perfused organs and therefore are an extremely important variable in determining the outcome of EVLP.

Steen *et al*[4,5,12] developed a new perfusion solution and proved EVLP to be a viable method to improve and preserve donor lungs, and it continues to be the most popular perfusion solution used. The Pego-Fernandes group reported that their solution, low potassium dextran-glucose (LPDnac) was comparable to Perfadex (Vitrolife, Goteborg, Germany) but found saline to be inadequate[13,14]. Menezes *et al*[15] also compared Perfadex to Celsior and found lungs perfused with either exhibited similar gas exchange and histopathological findings.

Gene or molecule delivery: Multiple groups have shown that gene therapy coupled with EVLP can repair injured lungs before transplantation. Cypel *et al*[16] demonstrated that the delivery of adenoviral vector encoding human interleukin-10 (AdhIL-10) to human lungs showed improvement in arterial oxygen pressure and vascular resistance, concluding that delivery of AdhIL-10 can improve lung function. Yeung *et al*[17] later showed that ex vivo delivery of adenoviral genes to lungs is superior to *in vivo* delivery due to the decreased vector-associated inflammation and improved post-transplant lung function. Emaminia *et al*[18] also showed that delivery of adenosine A2a in the perfusate reduced the inflammatory response in acutely injured pig lungs.

Optimize the nutrients needed to sustain the lungs: Using an acellular perfusate can avoid mechanical damage to the lung over long lung perfusions[1,19] and is more widely used over cellular solutions. Pro-inflammatory cytokines can accumulate in the perfusate over time so the perfusate should be replaced periodically to avoid increased inflammation.

Trans tracheal and aerosolized agent delivery: Drugs cannot only be delivered through the perfusate, but also as an aerosolized drug trans-tracheally. Pulmonary delivery of aerosolized drugs has been modeled using an EVLP system by many groups. Dong *et al*[20] showed that administration of aerosolized chitosan-coated poly (lactide-co-glycolide) based nanoplexes containing antisense 2'-O-Methyl RNA (OMR) resulted in a significantly higher uptake of OMR in the respiratory epithelium compared to administration of OMR alone using an EVLP model. Beck-Broichsitter *et al*[21] also used an EVLP model to show that delivery of biodegradable nanoparticles may be a viable approach for drug delivery.

LARGE ANIMAL MODEL OF EVLP

Advantages

Porcine EVLP has a direct translation to the human clinic (Figure 1). In general, the advantages of this large animal model of EVLP can be broadly grouped into the following 4 categories.

Table 2 Physiologic *ex vivo* lung perfusion parameters

	Rat	Pig
Tidal volume	4-10 mL/kg	6-8 mL/kg
Positive end expiratory pressure	2-6 cm H_2O	5 cm H_2O
Flow rate	5-30 mL/min (estimated cardiac output: 25-50 mL/min per 100 g)	40% cardiac output/min (estimated cardiac output: 100 mL/min per kilogram)
Pulmonary artery pressure	13.6 cm H_2O	10-15 mmHg
Perfusate albumin concentration	2%-4%	5%-7%

Size appropriate: The swine model offers very appropriate size comparisons to humans. Because of this, comparable tidal volumes, PEEP, and perfusion times can be used for the EVLP. As a result, information obtained in this large animal model of can be rapidly and directly transferred to settings for clinical trials. This direct transfer of information to clinical trials is typically not possible when using smaller animal models. There are variations in physiologic parameters based on animal model sized (Table 2).

Similar immune system and biology: The pig has a greater similarity to humans in gene sequence and physiology compared to mice and rats which makes it a superior model[22]. This results in a simpler comparison to humans and therefore a more direct path to clinical relevance.

Allows for opportunity to perfect scale up to human size and clinic setting: Because of the pig's larger size, the opportunity exists to experiment with the exact same equipment that would be used in clinical trials[12]. The amounts of perfusate needed as well as ventilator settings are more closely related to clinical settings compared to smaller animal models. The amount of time a pig lung can be perfused is comparable to humans.

Accepted transplant model: All animals should receive care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, the Guide for the Care and Use of Laboratory Animals.

Generally, protective settings for mechanical ventilation are used during EVLP. Typically, pigs are sedated with 40 mg/mL ketamine and anesthetized with 8 mg/kg pentobarbital. A period (about 60 min) of warm ischemia is usually implemented before starting EVLP to mimic a donor's lung. Volume-controlled ventilators are used and tidal volumes of about 4-6 mL/kg, a PEEP of about 5 cmH_2O, and a respiratory rate of about 17 breaths per minute are used. The fraction of inspired oxygen (FiO_2) usually ranges from 100% to 40% (Table 2).

Limitations

While pigs offer one of the best parallels to humans, their cost to purchase and to care for is much higher than small animals. The amount of perfusate used on an isolated pig lung is much higher than with small animal, making each experiment much more expensive. This also makes having a high amount of replicates in an experiment very difficult. Because of the large size of an isolated pig lung, the perfusion circuit itself is custom built and requires the same equipment that would be used for a clinical perfusion.

SMALL ANIMAL MODEL OF EVLP

Overall advantages

Small animal models that have been employed in EVLP include rat, mouse, guinea pig and rabbit (Figure 2). These systems offer several distinct advantages compared to their larger counterparts. Overall, their cost is much lower. This includes initial startup cost, such as in surgical and perfusion equipment as well as the animals themselves. Because of the smaller cost, one can complete more perfusion experiments with less money and in less time than if the same study were completed using a porcine or canine model system. Additionally, one can capitalize on the higher sample size in order to aid in achieving statistical significance. Most small animal experiments use 5-8 animals per group and up to 50 animals in total per study[23-26]. These numbers are simply not feasible in larger systems and helps increase confidence in experimental data.

An inherent advantage of EVLP is the isolation of the lungs from the rest of the body. This has helped elucidate differences in the immune response of resident lung cells compared to the systemic immune response during ischemia/reperfusion (I/R) in a mouse model[26,27]. More generally, this characteristic of EVLP can be exploited to more easily vary experimental components and limit confounding factors. One avenue of research that has been pursued extensively is in the optimization lung perfusate solutions. This is an area of critical importance in the development and refining of EVLP procedures for clinical use and is a current topic of controversy.

Basic properties, such as the optimal electrolyte composition of the perfusate itself are not agreed upon. Current data are unclear as to which currently available solutions perform best[15]. Perfadex, a solution developed specifically for lung preservation, may not offer better preservation than Celsior, a heart preservation solution[15,25]. One group in Brazil compared Perfadex to a locally produced generic solution, LPDnac and found it to preserve lungs just as well[13]. The potential benefits of varying perfusate temperature and introducing vasodilators has also been studied[28]. Despite the disagreements over perfusate composition, small animal EVLP systems provide an excellent platform for further perfusate development and testing.

Rat/rabbit/guinea pig models

Of the different small animal systems used for EVLP experiments, each offers their own advantages and draw-

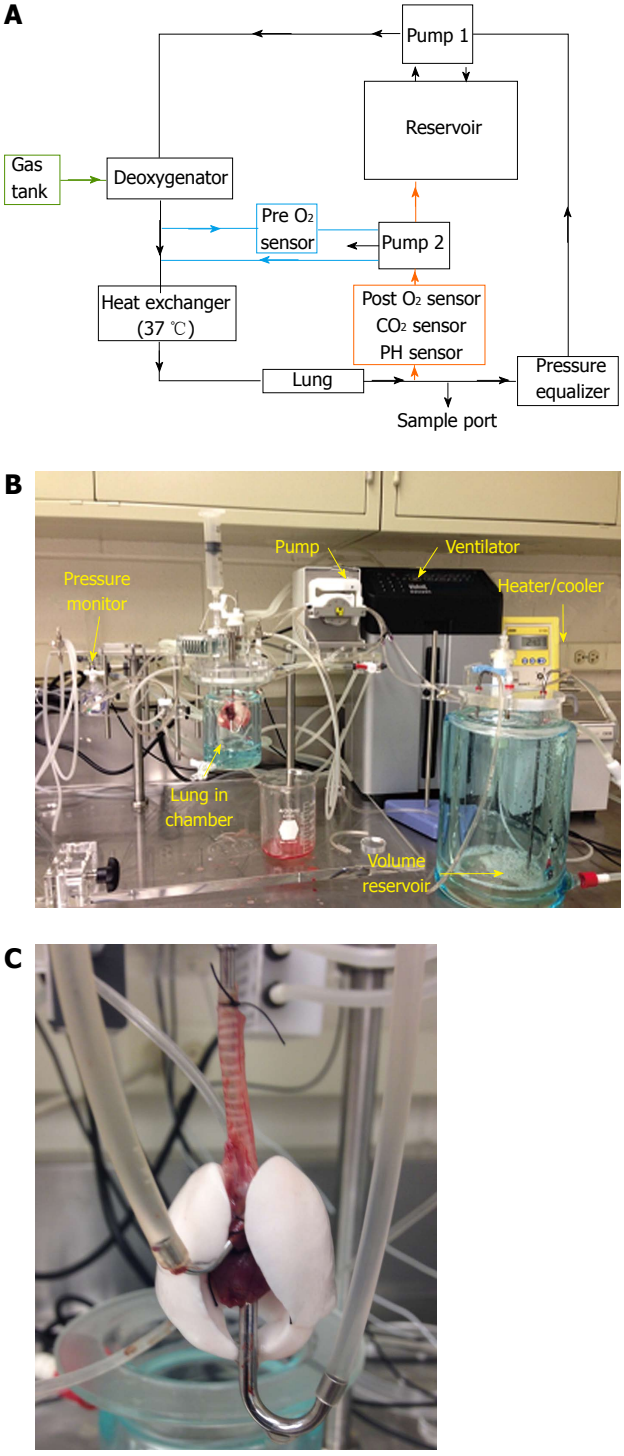

Figure 2 This diagram depicts a schematic of a small animal (rat) *ex vivo* lung perfusion circuit (A), the small animal perfusion circuit (B); a close-up of a rat lung undergoing *ex vivo* perfusion (C). Many of the same characteristics that are in the large animal circuit are present. This particular circuit has the ability for fine measurements of pressure, flow, and weight. The image back right shows the thermoregulator and the ventilator. The perfusates reservoir is in the image front right. The small animal circuit is analogous to the large animal circuit. However, due to the relative scale of the organ to the circuit, the perfusate volume needed for a complete perfusion is less. In addition, the ability to perform positive as well as negative pressure ventilation is possible. This varied ventilation an mimic both the mechanical breathing as well as natural intrathoracic breathing. The tracheal cannulation is top-center. The inflow cannula going into the pulmonary artery is from top-left and the outflow cannula going across the left atrium through the left ventricular apex is on screen right.

backs. Rat, guinea pig and rabbit models have a larger thoracic cavity than mice, making surgical procedures easier. Owing to their larger size, initial cannulation (Figure 2) is relatively simple and can be done with or without the aid of a surgical microscope[29,30]. Moreover, a rat left lung transplantation (LTX) technique has been developed and used in multiple studies[29-32].

Recent improvements have increased the success rate of this LTX technique to greater than 95%[29]. Inokawa *et al*[32] used this procedure to create a specific model of transplantation as it relates to EVLP and designed it to closely mimic clinical conditions. Rat donor lungs are explanted, stored on ice for 1 h, perfused, stored on ice again for 2.5 h and finally transplanted. This model has been used to demonstrate the therapeutic potential of low concentration carbon monoxide ventilation during perfusion[23]. Although less common, rabbit[33] and guinea pig[34] models have been used to study the onset of ischemia-reperfusion injury.

One challenge, however, with the use of these three animals as model systems is the relative scarcity of species-specific commercially available antibodies and molecular reagents. Because of this, protein studies are limited in these systems, though Fehrenbach *et al*[35] demonstrated in a rat model of EVLP that the concentration surfactant protein A (SP-A) increased following I/R using a polyclonal antibody against SP-A.

Murine models

Murine models of EVLP offer considerable advantages over rats because of the greater number of species-specific antibodies and gene probes available for experiments. This has facilitated development of a much greater body of scientific literature with regard to these types of studies. For example, the murine immune response to EVLP has been studied for over 15 years[36]. More recently, Barrenschee *et al*[37] used toll like receptor (TLR) agonists to mimic the response during infection and characterized levels of key cytokines/chemokines such as interleukin (IL)-1β, IL-6 and TNF-α. Siegl and Ulrig studied the inflammatory response of mice in high and low ventilation scenarios, including quantification of the phosphorylation of key enzymes involved in the inflammatory response[38].

An additional advantage of the mouse model is the availability of knock out (KO) lines. Deficient genes could be related to the inflammatory response, including TLR-4 deficient[39] and TNF-α deficient mice[27] or could interfere with other areas of lung function[40]. Maxey *et al*[27] used the TNF-α deficient mice in EVLP to demonstrate the importance of TNF-α in initiating the inflammatory response following I/R.

Recently, a model of mouse lung transplantation has been developed for further study of obliterative bronchiolitis. The procedure is very similar to the rat model of LPX from a technical standpoint, but to our knowledge, has not yet been used as an EVLP model[41,42]. This may be due to increased technical difficulties during mice op-

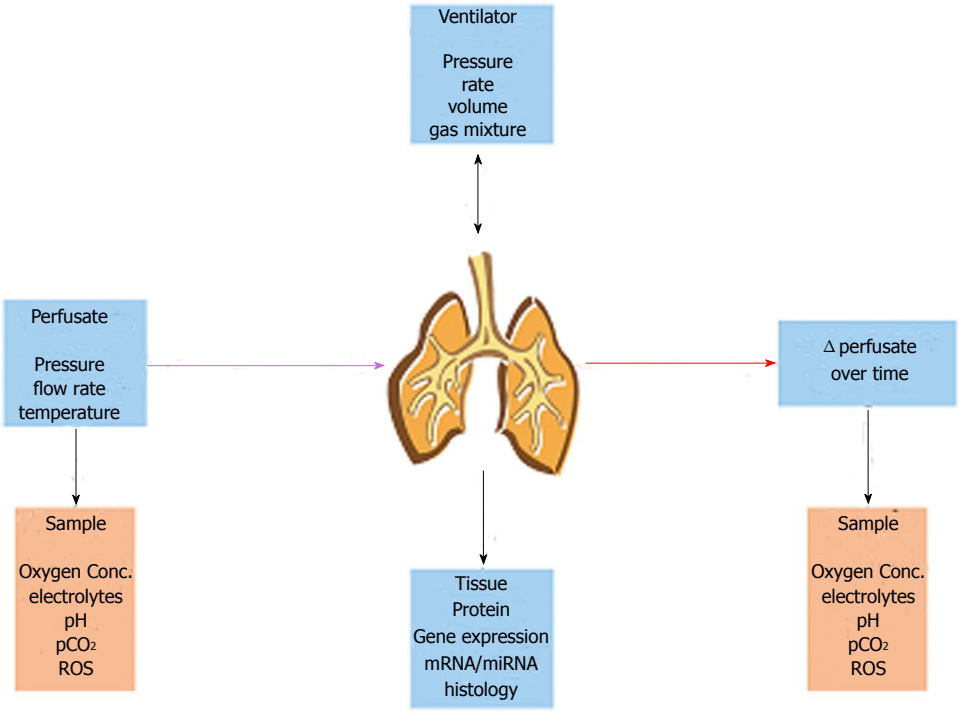

Figure 3 Diagram of what is able to be able to be measured and varied with the *ex vivo* perfusion circuit. This figure directly correlates with Table 1.

erations because of their smaller size. However, it is likely that once some initial sets of experiments combine this mouse LTX technique with EVLP, the scope of possibilities of what EVLP platforms can study will be widened.

The greatest challenge in solely relying on the murine model of EVLP is the technical difficulties involved during surgery. Mice have a smaller thoracic cavity and smaller organs than rabbits, rats or guinea pigs. Often, a surgical microscope is required to identify and isolate key structures during the heart-lung block explant[26,27]. Another drawback of this mouse model is the increased difficulty of training personnel on more technical mouse surgery procedures, which can create bottlenecks in experimental plans and ultimately slow down data acquisition. For this reason, it is likely that future studies will still utilize all small animal models, with mouse models of transplant used when necessary (for protein and gene studies) and lung mechanics studies primarily completed using a rat model.

Limitations

Owing to their small size and cost effectiveness, small animal models of EVLP are extremely convenient. When considering their use, however, several key differences need to be taken into account. Mice and rats have much shorter perfusion times than human or pig lungs. One rat model of lung transplant includes 15 min of perfusion time[32]. Other studies perfuse for 50 min[15,25,35] or 60 min[15]. One needs to keep in mind the mismatch in times scales, as murine lungs after 15 min of perfusion/ventilation are closer in damage to pig/human lungs perfused for a much longer time (4-24 h depending on the lung injury model being studied).

Yet another difference is that rodent lungs are significantly more susceptible to atelectasis. As a result, during the "ischemic" periods of a mouse model of EVLP, the lungs are still ventilated, albeit at a lower rate and in a hypoxic environment[26,27] This is unavoidable though, since without ventilation the lungs would not remain viable long enough to complete the study. Previous investigators have demonstrated that atelectasis and the subsequent reopening of fluid occluded regions can damage the lung epithelium[43,44] and exacerbate inflammation[45,46]. Therefore, it is extremely important to prevent lung damage, atelectasis and pulmonary edema because, unlike human and large animal models, a bronchoscopy cannot be performed to clear fluid from the lungs. Assuming all of these major differences are taken into account, small animal models are excellent starting points for the development of EVLP for clinical use and for the testing of therapeutics against I/R injury.

TECHNICAL CONSIDERATIONS

Perfusate

Steen solution is the most popular solution used to date and acellular solutions are much more common than cellular solutions. Studies indicate a hyper-oncotic, albumin-based solution is best. The acelluar solutions have the potential benefit of not adding an exogenous antigen source and the red cells are not lysed through the mechanics of the perfusion. The acellular solutions have the potential benefit of helping to support metabolic demands. In the lung this is not as critical as in other organs since the lung itself provides the oxygen. The perfusate needs to be buffered and provide glucose and electrolytes.

Ventilator settings

Ventilator settings should be protective during EVLP for best results. In the large animal model this means a tidal volume of 4-6 cc/kg. From time to time, 10 cc/kg is used. In the rat model, a protective tidal volume is 4 cc/kg with 10 cc/kg being potentially deleterious. Depending on the hypothesis being tested and the animal model used, multiple variables can be changed on the ventilator including tidal volume, PEEP, breaths per minute, and fraction of inspired oxygen (Figure 3).

Temperature

Perfusate temperature is usually either increased temporally or based on current temperature. The perfusate temperature is usually increased until 37 ℃ is achieved. An in-line thermoregulator or perfusion heater/cooler is used to titrate the temperature. A cold or warm ischemic period may precede the actual perfusion depending on the hypothesis being tested.

Duration of perfusions

Small animal perfusions usually run between 30 min-3 h. Pig EVLP have been run for up to 14 h. The times vary greatly depending on the animal model used and the hypothesis being tested.

Pulmonary artery flow rates and pressures

Perfusate flow rates are usually set to achieve a specific pulmonary pressure or a specific pulmonary resistance. A typical experimental set-up is to have the perfusion flow rate increase incrementally over the duration of the perfusion (15-30 min time period). Once full flow (40% cardiac output) is achieved, the pulmonary artery and left atrial pressures are measured. The pulmonary vascular resistance is calculated as a function of the pressures and flow rates. In a well-functioning organ, pulmonary vascular resistance decreases over time. In a poorly functioning organ, the resistance increases. Increased resistances often mirror poor oxygenation.

CONCLUSION

EVLP has great potential to increase the lung donor pool by providing a platform for improving and evaluating lungs initially thought to be inadequate. Multiple groups across the globe are developing promising models to achieve a greater donor pool. EVLP is also being used as a model for acute lung injury to better understand how the complex mechanical forces applied to the lungs influence injury development and inflammation and to develop strategies that limit the amount of tissue damage/inflammation. EVLP is also being explored as an opportunity for administering therapeutic agents. This idea is unique in that it bypasses the patient's immune system and allows for a higher acceptance rate compared to drugs administered *in vivo*.

Both small and large animal models are advancing our knowledge on EVLP and each has their own specific advantages and disadvantages. While small animal models do not usually run for more than 1-2 h, they are economical and allow for many experiments in a short period of time. Swine models are very expensive but allow for the closest model to human lungs available and use the same equipment that would be used clinically. Since nearly 50% of patients die while waiting for a lung transplant, it is crucial to expand the donor pool. EVLP holds the most promise towards achieving this goal.

The ability to keep organs alive and perfused for extended periods of time will enable the "culture" of organs. This prolonged, perfusion will be the basis for immunomodulation and change of the endothelium through nanoparticle, gene bases, or antibody based delivery of therapeutic agents. This will be the dawning of customized medicine to tailor the transplanted organ to the individual recipient and their biology.

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MINIREVIEWS

Wound healing reaction: A switch from gestation to senescence

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Abstract

The repair of wounded tissue during postnatal life could be associated with the upregulation of some functions characteristic of the initial phases of embryonic development. The focusing of these recapitulated systemic functions in the interstitial space of the injured tissue is established through a heterogeneous endothelial barrier which has excretory-secretory abilities which in turn, would induce a gastrulation-like process. The repair of adult tissues using upregulated embryonic mechanisms could explain the universality of the inflammatory response against injury, regardless of its etiology. However, the early activation after the injury of embryonic mechanisms does not always guarantee tissue regeneration since their long-term execution is mediated by the host organism.

Key words: Wound healing; Repair; Embryonic mechanisms; Vitelline; Amniotic

Core tip: In this review, we propose an integrative molecular point of view about wound healing. Wound healing could be associated with the upregulation of functions characteristic of embryonic development. The repair of adult tissues using upregulated embryonic mechanisms could explain the ubiquity of the inflammatory response against injury, regardless of its etiology.

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INTRODUCTION

Wound tissue repair can be realized by regeneration and/or fibrosis. While regeneration describes the specific substitution of the injured tissue, tissue fibrosis displays an unspecific form of healing in which the wounded tissue heals by scar formation[1,2]. Since repair by fibrosis can be considered an unsuccessful attempt of wound tissue repair by regeneration, the fibrotic process supposedly represents an insufficient repair method and, therefore, a pathological response. This is the reason why the inflammatory response associated with scar formation is also commonly labeled pathological. In this way, regenerative healing has a notable absence of inflammatory cell activity[3-5]. Consequently, inflammatory response mediators have been a focus of investigation in studies aiming to curtail scarring[5,6].

The standard view of inflammation as a reaction to injury or infection might need to be expanded to account

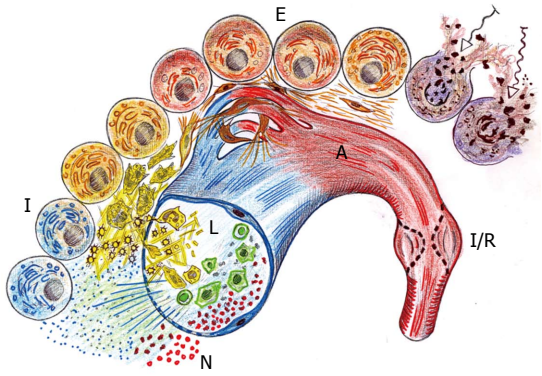

Figure 1 Schematic representation of the different stages of wound repair. During the post-traumatic local inflammatory response three successive and overlapped phases: in the arterial side of the microcirculation (red), a nervous (N) or immediate phase with ischemia-reperfusion (I/R) occurs; in the post-capillary venule (blue), an immune (I) or intermediate phase with a leukocytic (L) phenotype is expressed; and, finally an endocrine (E) or late with an angiogenic (A) phenotype is developed, which implies the capillaries neoformation.

for the inflammatory processes induced by other types of adverse conditions[7]. The human diseases that are associated with these conditions, including atherosclerosis, asthma, type 2 diabetes and neurodegenerative diseases, are all characterized by chronic low-grade inflammation[7]. However, human aging can be explained by the emerging concept of inflamm-ageing, *i.e.*, - a combination of inflammation and aging[8]. Inflamm-ageing seems to favor the onset of typical age-related diseases like atherosclerosis, dementia, osteoporosis and cancer[9]. Inflammatory mechanisms are also involved in physiological processes, like physical exercise, embryonic development and gestation, and indeed there is the hypotheses that the evolution of the living species could be based on inflammatory remodeling of organisms induced by environmental factors[10]. It has also been proposed that, although fibrosis is often initially linked to a strong inflammatory response, there are specific mediators and pathways contributing to the pathogenesis of fibrosis that are distinct from the mechanisms driving inflammation. Thus, it is assumed that to design effective therapy for fibrotic diseases, we need to begin viewing fibrosis as a pathological process distinct from inflammation[11].

PHASES OF THE SKIN WOUND HEALING REACTION

The multiple pathophysiological mechanisms that overlap during the progression of the skin wound healing reaction may explain the lack of consensus on the number of phases involved in this reaction. Thus, the common description of the wound healing evolution includes three classical stages: the inflammatory phase to contain the injury and prevent infection; the proliferative phase characterized by new tissue formation, *i.e.*, granulation and epithelial tissues; and the remodeling phase with extracellular matrix reorganization[4,12]. However, some authors describe four healing phases: hemostasis and coagulation, with the formation of a provisional wound matrix; inflammation with neutrophil and monocyte recruitment; proliferation and repair, with the formation of granulation tissue and the restoration of the vascular network, as well as re-epithelialization; and remodeling that occurs from day 21 to up to 1 year after injury. In this phase, collagen Ⅲ, which was produced in the proliferative phase, is now replaced by collagen I and the acute wound metabolic activity slows down and finally stops[1,13]. Additionally, five phases of the wound healing reaction have also been described: hemostasis; inflammation; cellular migration and proliferation; protein synthesis; and wound contraction and remodeling[14].

In the above-mentioned descriptions of the wound healing reaction, the role attributed to inflammation is very limited and noteworthy. On the contrary, we have proposed an inflammatory etiopathogenic hypothesis of the wound healing evolution. According to this idea, inflammation could be the basic mechanism that drives the nature of the different stages of wound repair[15]. Likewise, inflammation could facilitate the integration of the pathophysiological mechanisms involved in the different phases of wound repair by scar formation[15,16]. In essence, the post-traumatic local acute inflammatory response is described as a succession of three functional phases of possible trophic meaning to the wounded tissue: nervous or immediate with an ischemia-reperfusion phenotype; immune or intermediate with a leukocytic phenotype; and endocrine or late with an angiogenic phenotype[15,16] (Figure 1).

In turn, we have suggested that these phenotypes could represent the expression of trophic functional systems of increasing metabolic complexity[17]. Therefore, it could be considered that, after the injury, the metabolic ability of every phenotype would be conditioned by the biochemical mechanisms used to provide the energy sources for cell functions[15,17]. These three inflammatory phenotypes hypothetically expressed in the traumatized tissue during tissue repair by scarring could help to integrate the etiopathogenic mechanisms expressed in each evolutive phase. In this way, these inflammatory phenotypes would associate the genetic factors, upregulated and/or downregulated, with metabolic, functional and histological alterations[17].

The interstitial space is the battle field where the inflammatory response takes place. In the successive phases of the inflammatory response, the interstitial space of the injured tissues is successively occupied by molecules, inflammatory cells, bacteria and finally by a mesenchymal-derived tissue, the granulation tissue. In summary, the inflammatory response could be viewed as a series of three overlapping successive phases with increasingly complex trophic functional systems for using oxygen since it evolves from ischemia to neovascularization[15,17].

The first or immediate phase has been referred to as the nervous phase because sensory (stress, inflammatory, pain and analgesia) and motor (contraction and relaxation) alterations, including vasomotor changes, respond

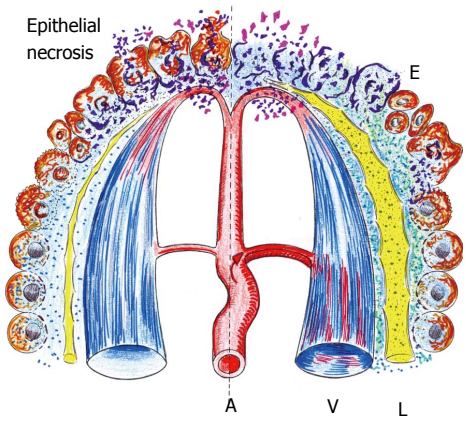

Figure 2 First or immediate phase of the acute inflammatory response. On the left side, a schematic representation in which the tissue suffers the injury and therefore necrosis of the epithelial cells are produced. In turn, on the right side, the beginning of the tissue inflammatory response in response to necrosis is shown. This initial phase presents ischemia-reoxygenation and interstitial edema (E) with interstitial infiltration of mediators of the stress response as well as substrates including glucose, amino acids and lipids. In addition, the lymphatic circulation (L) is activated. A: Arterial microcirculation; V: Post-capillary venous circulation.

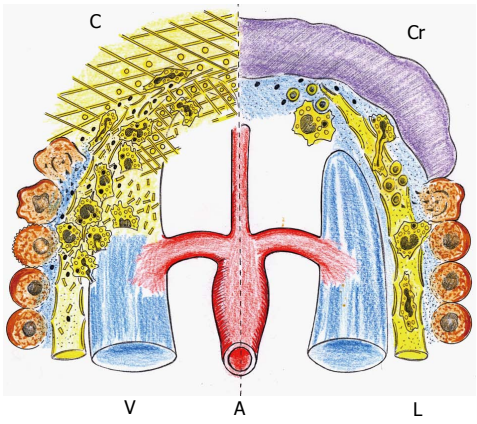

Figure 3 Immune or intermediate phase of the post-traumatic acute inflammatory response. Interstitial infiltration by platelets and leukocytes, all of them entrapped in the provisional extracellular matrix (left). Underlying the wound crust (Cr) that is formed later, the leukocytes change their phenotype to promote the resolution of the inflammatory response and wound repair by re-epithelization and scar formation (right). C: Coagulation with fibrin-platelet clot. A: Arterial microcirculation; V: Post-capillary venous circulation; L: Lymphatic circulation.

to the injury. This early pathological activity of the body's nociceptor pathways is associated with stress through the hypothalamic-pituitary-adrenal and sympathetic-adrenal medullary axes, the sympathetic nervous system and the renin-angiotensin-aldosterone system. This initial phase presents ischemia-reoxygenation, oxidative and nitrosative stress, and interstitial edema with selective interstitial infiltration by mediators of the stress response, such as catecholamines, adrenocorticotrophic hormone, glucocorticoids and angiotensin, as well as glucose, amino acids and lipids, all of them derived from earlier metabolic alterations, including hyperglycemia, protein catabolism and lipolysis. In addition, interstitial edema favors nutrition by diffusion through the injured tissue and activation of the lymphatic circulation (circulatory switch)[2,15,17] (Figure 2).

In the succeeding immune or intermediate phase of the acute inflammatory response, the wounded tissue that has previously suffered ischemia-reperfusion is infiltrated by inflammatory cells and sometimes by bacteria. This phase presents enzymatic stress with migration of macrophages and dendritic cells to lymph nodes, where they activate T and B cells, *i.e.*, innate and adaptive immune response. Interstitial invasion by leukocytes would create a new trophic axis.

Accumulating evidence demonstrates that platelets contribute to the initiation and propagation of the inflammatory process. These cells are replete with secretory granules, α-granules, dense granules and lysosomes. Platelet α-granules influence inflammation both by expressing receptors that facilitate adhesion of platelets to other vascular cells (*e.g.*, P-selectin) and by releasing a wide range of chemokines, among which CXCL4 and CLXL7 are the most abundant. Also, platelet α-granules contain a variety of both pro- and anti-angiogenic proteins. Growth factors stored in α-granules include vascular endothelium growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor and insulin-like growth factor (IGF). Platelet dense granules, on the other hand, contain high concentrations of low molecular weight compounds that potentiate platelet activation (*e.g.*, Adenosine diphosphate, serotonin and calcium[18,19] (Figure 3).

In the post-traumatic local inflammatory response, the activation of the innate immune system is not only based on the recognition of danger signals or danger-associated molecular patterns (DAMPs), but also relies on the presence of pathogen-associated molecular patterns (PAMPs)[20]. DAMPs and PAMPs are recognized by pattern-recognition receptors (PRRs) that are either cytoplasmic, membrane-bound or secreted. The most intensely studied PRRs are the Toll-like receptors (TLRs), in addition to innate immune receptors, the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)[21]. In particular, NLRs form central molecular platforms that organize signaling complexes, such as inflammasomes and NOD signalosomes. The term inflammasome was coined to describe the high molecular weight complex that activates inflammatory caspases and cytokine interleukin-1 (IL-1)β[22]. All these receptors activate signaling cascades that is based on enzymatic intra- and extra-cellular digestion[15,17] and lead to activation of mitogen activated protein kinases and nuclear factor kappa B (NF-κB)[21,22]. Once activated, TLRs induce different signaling cascades depending on the adaptor protein, ultimately leading to the activation of the transcription factors NF-κB, AP-1 and interferon-regulatory factor[22]. The regulatory event of NF-κB activation is the phosphorylation of inhibitor of kappa B kinase complex (IKB) proteins by the IKB kinase

complex, which leads to IKB protein ubiquitylation and subsequent degradation. This results in the release of cytoplasmic NF-κB complexes, which then translocate to the nucleus and drive the expression of target genes[23]. Thus, the expression of inducible genes leading to the synthesis of cytokine receptors, adhesion molecules and autacoids in the traumatized tissue is induced[24].

Leukocytes transverse the subendothelial basement membrane during their immunological surveillance patrol through tissues. This process, called diapedesis, is strongly enhanced under the influence of inflammation. The preferred extravasation sites of leukocytes are the venules[25]. Immediately after injury, extravasated neutrophils are entrapped in the fibrin-platelet clot. In the interstitium, the recruited and activated neutrophils begin the debridement of devitalized tissue and attack infectious agents. To perform this task, they release a large variety of active antimicrobial substances (ROS, cationic peptides, eicosanoids) and proteases (elastase, cathepsin G, proteinase 3 and urokinase-type plasminogen activator)[12]. Neutrophils also store pentraxins 3 and release it in response to inflammatory signals because it is an acute phase reactant[26] (Figure 3).

As monocytes extravasate from the blood vessel they become activated and differentiate into mature tissue macrophages. This transformation implies major changes in gene expression and cell function. The differential activation of macrophages is involved in many facets of tissue injury and inflammation. M1 macrophages express pro-inflammatory cytokines, such as IL-1, IL-6, IL-23 and interferon (IFN)-γ, as well as reactive oxygen and nitrogen species, which are involved in phagocytosis and the killing of microbes. They also promote type I immune responses[27]. M2 or alternatively activated macrophages fail to express pro-inflammatory mediators and are involved in angiogenesis, tissue remodeling and the resolution of inflammation. Therefore, they are supposed to promote repair functions[12,27]. T-helper cells play critical roles in modulating the differential activation of type 2 macrophages. T-helper (Th1) cells produce pro-inflammatory cytokines, *i.e.*, IFN-γ and TNF-α, which skew macrophages into the M1 phenotype. In contrast, type 2 T-helper (Th2) cells produce IL-4, IL-5, IL-13 and IL-10, which are responsible for inducing the alternatively activated macrophages or M2 macrophages[28]. Finally, it has been speculated that metabolic changes in the local milieu may program dendritic cells and other innate cells at the site of inflammation to induce a heterogeneous Th2 response[29]. Although neutrophils, macrophages and T lymphocytes are considered central in the pathogenesis of post-traumatic inflammation, recent studies also imply the involvement of mast cells and B lymphocytes as modulators of the inflammatory response and wound healing[12,30].

In the final and lasting phase of the wound healing reaction, the angiogenic phenotype is predominant because angiogenesis permits numerous substances, including hormones, to be transported by the blood circulation. Angiogenesis is based on endothelial sprouting or intussusceptive (nonsprouting) microvascular growth[31]. However, angiogenesis can also result from the recruitment of several cell populations or selected subpopulations of bone marrow-derived endothelial progenitor cells[32]. Angiogenesis is regulated by numerous "*classic*" factors, including VEGF, FGF-2, transforming growth factor (TGFs) angiopoietins, PDGF, thrombospondin-1 and angiostatin. Non-classic endogenous stimulators of angiogenesis include erythropoietin, angiotensin Ⅱ, endothelins, adrenomedulin, adipokines, neuropeptide-Y, vasoactive intestinal peptide and substance P[31]. VEGF and FGF-2 occupy the center stage in the angiogenesis field. They act in synergy to stimulate endothelial cell function during angiogenesis in tissue repair[33]. In this last phase, the endocrine phenotype favors nutrition mediated by the blood capillaries. Through initial and excessive proliferation, the endothelial cells could play a key role in the previous phase as antioxidant and anti-enzymatic cells, including induction of the acute phase response, considered the humoral arm of innate immunity[15,16]. Angiogenesis is closely associated with granulation tissue formation and remodeling. As granulation tissue forms in the healing wound, the vascular cells intermingle with the provisional matrix, which is composed mainly of fibrin, fibronectin and vitronectin[33]. Then, the new blood vessels associated with fibroblasts and macrophages replace the fibrin matrix with granulation tissue, forming a new substrate for keratinocyte migration[34] (Figure 1).

The resolution of the inflammatory response is mainly mediated by families of local-activity mediators that are biosynthesized from the essential fatty acids eicosapentaenoic acid and docosahexaenoic acid. These resolution mediators are termed resolvins, maresins and protectins[35]. Inflammation resolution is also mediated by lipoxins that are generated through platelet-leukocyte interactions[36] (Figure 3). It has been also proposed that regulatory T cells (Treg cells) have evolved to provide a complementary immunological arm to a physiological tissue-protecting mechanism driven by low oxygen tension, *i.e.*, hypoxia, in the inflamed tissues. The hypoxia-adenosinergic pathways might govern the production of immunosuppressive molecules that have already been implicated in the activities of Treg cells[37]. In this way, Treg cells could exert their suppressive function with local downregulation of immune response, inducing "*immunodormancy*" and protecting tissues from collateral tissue damage, thus improving healing[37]. The progressive resolution of inflammation favors wound re-epithelization. Fibroblasts can also contribute to the resolution of inflammation by withdrawing survival signals and normalizing chemokine gradients, thereby allowing infiltrating leukocytes to undergo apoptosis or leave the tissues through the draining lymphatics[38]. Remodeling begins two to three weeks after injury and lasts for a year or more. Most of the endothelial cells, macrophages and myofibroblasts, undergo apoptosis, leaving a mass that contains few cells and consists mostly of collagen and other extracellular-matrix proteins[34]. However, the prognosis of extensive and deep wounds is not entirely satisfactory because of

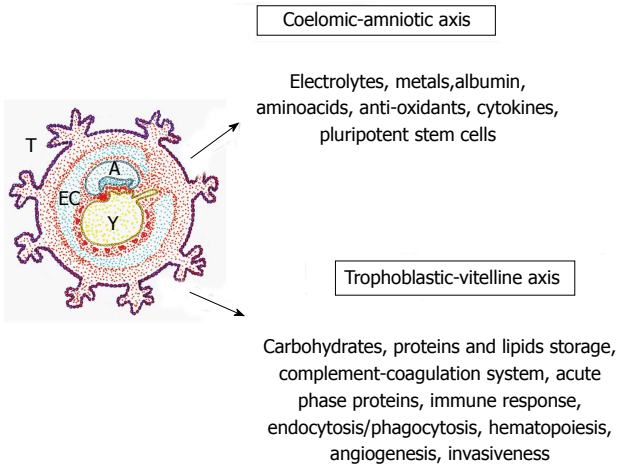

Figure 4 Hypothesized functions by ontogenic recapitulation in the traumatized tissue. These functions could be similar to the extra-embryonic coelomic-amniotic and trophoblastic-vitelline functions during early embryonic development. The extra-embryonic coelom or exocoelomic cavity surrounds the blastocyst, which is composed of the amnion and the primary yolk sac. EC: Exocoelomic cavity; A: Amnion; T: Trophoblast; Y: Yolk sac or vitellum.

scar formation and loss of normal function and skin appendages. Therefore, reducing the formation of scars and re-establishing the normal anatomy and function of the skin and its appendages have become the aim of regenerative medical research[39,40].

WOUND HEALING REPAIR USING EMBRYONIC MECHANISMS

Inflammation, whether acute or chronic, produces tissue remodeling[9]. In this way, it has been proposed that the inflammatory response has features in common with tissue development, which requires involution of pre-existing tissue elements[15,16]. The ability of the tissues to involute or dedifferentiate could represent a return to early stages of development[41]. Particularly, involution or dedifferentiation could form an effective defense mechanism to escape death after injury. Thus, this mechanism could make retracing an ancient, efficient and well-known route possible for repairing the injured tissue, just like the initial phases of embryonic development[2,41]. The correlation that can be established between the embryonic and the inflammatory events suggests that the results obtained from research into both great fields of knowledge would favor each other and promote their development[41].

In the adult body, many pathways that play an essential role during embryological development are inactivated later in life, although some of them may be transiently expressed during the adult repair process[41,42]. This ability of the tissues to involute or dedifferentiate could constitute an effective solution against any type of injury. Through dedifferentiation, tissues have the chance to reform and remodel themselves according to the new environmental situation imposed on them[10].

The fetus is uniquely capable of healing skin wounds without scar formation and provides a model of ideal tissue repair. Understanding the biology of this process may allow us to modulate wound healing in children and adults to become more fetal-like[43-45]. Tissue repair in the embryo and to a certain extent in adults too, appears to recapitulate those cell machineries used by embryos to undergo the natural tissue movements of morphogenesis, such as gastrulation and neural tube closure[41,46]. One key difference between embryonic and adult repair, which may explain why one heals perfectly and the other scars, is the presence of an inflammatory response at sites of adult repair while there is none in the embryo. However, total knockdown of inflammation is clearly not going to be an optimal treatment for post-natal scarring[46]. The infiltration of platelets, mast cells, neutrophils and macrophages which characterizes the early postnatal wound is greatly diminished in fetal wounds[43,47]. However, fetal wound healing is additionally characterized by a distinct extracellular matrix, anti-inflammatory and growth factor profile and a more important role for stem cells[5,6]. If so, we could hypothesize that to promote adult wound repair by regeneration, current therapies need to be attempted to recapitulate singular aspects of the fetal regenerative phenotype[5]. The evidence suggests that there may be an early critical window in postnatal wound healing that may be amenable to manipulation so as to provide a permissive environment for scarless wound healing to proceed[5].

In this way, the early post-traumatic inflammatory response could recapitulate ontogeny by re-expressing two hypothetical extra-embryonic trophic axes, that is amniotic and yolk sac or vitelline in the interstitial space of the injured tissue[41] (Figure 4). Likewise, the body could be repaired according to embryonic biochemical patterns through the expression of extra-embryonic functions. If so, the early inflammatory steps could represent the postnatal debut of ancestral biochemical mechanisms that were used for normal embryonic development. The re-expression of these ancient mechanisms is perhaps hard to recognize because they are anachronistic during postnatal life and are established in a different environmental medium[41,48] (Table 1).

After fertilization, the first stage of embryogenesis is the zygote, which undergoes cleavage by mitosis. When the morula stage is reached, the embryo establishes polarity. The cells bind tightly to each other, forming a compact sphere with two cell layers. The outer most layer becomes the trophoblast, giving rise to the placenta, and the inner cells become the inner cell mass, giving rise to the embryo and the remaining structures, including the amnion, yolk sac and allantoids[49] (Figure 4). The molecular and cellular contributions of the extra-embryonic tissues surrounding the fetus, namely the exocoelomic cavity, the amnion, the trophoblast and the yolk sac, to the interstitial space located between them, the mesoderm, are essential for organogenesis. In fact, the intra-embryonic mesoderm generated during gastrulation may represent the internalization of the functions that charac-

Table 1 Upregulation of extraembryonic phenotypes that could be involved in the different types of the wound healing reaction

Phenotypes	Embryonic functions	Phases of the inflammatory response	Phases of the wound healing reaction	
Extraembryonic phenotypes	Coelomic-amniotic axis	Nervous phase	Neurogenic systemic response	Stress response - Biogenic amines release Sensitive and motor alterations Ischemia-reperfusion - Local oxidative and nitrosative stress Hydroelectrolytic alterations - Edema Inflammation blood cells - Coagulation
	Trophoblastic-vitelline axis	Immune phase	Bone-marrow related response	Enzymatic stress Corticosuprarenal hormones - Local storage Hematopoietic stem cells Mesenchymal stem cells Endothelial progenitor cells
Embryonic phenotypes	Gastrulation	Angiogenic phase	Remodeling response	Myofibroblasts Angiogenesis Endothelial egg Re-epithelization Fibrosis

terize these extra-embryonic functions[50].

The hypothetical recapitulation of these initial phases of the embryonic development during the early surgical inflammatory response would imply the expression of functions similar to the extra-embryonic structures. Accordingly, the phenotype that could be adopted by the inflamed interstitium may induce the accumulation of fluid with similar characteristics to coelomic fluid. In essence, interstitial edema with high levels of proteins, in particular albumin, as well as electrolytes, metals, amino acids, antioxidants, cytokines and cholesterol-derived hormone, would be produced in the inflammatory exudates[51,52]. Amnion-derived multipotent progenitor cells also secrete a unique combination of cytokines and growth factors called the "*amnion-derived cellular cytokine solution*" which establishes a connection between mesenchymal and epithelial cells during embryo development[53]. In this sense, the amniotic fluid surrounding the fetus may therefore be an extension of the extracellular space of the fetal tissues[54]. The amniotic-like phenotype could also offer the stem cell a hypoxic and hydrated interstitial axis with cytokines and growth factors, favoring not only nutrition by diffusion, but also transport, excretion and bacteriostatic and anti-inflammatory protection[54,55] (Figure 4).

The wall of the secondary yolk sac is formed by an external mesothelial layer, a vascular mesenchyme, with blood islands that promote the development of hematopoiesis and angiogenesis[56] and an endodermal layer facing the yolk sac cavity[53]. The mesothelial and endodermal layers have absorptive functions and are active in endocytosis/digestion[56,57]. In addition, the endodermal layer is the source of several proteins including acute phase proteins[58]. A major function of the yolk sac is carbohydrate, protein and lipid accumulation for embryo nutrition (*vitellum*)[57]. In addition, through the synthesis and release of acute phase proteins, this extra-embryonic phenotype reduces oxidative, nitrosative and enzymatic stress, activates the complement-coagulation system, regulates the lipid metabolism and favors phagocytosis[59]. During trophoblast differentiation, trophoblastic cells also exhibit intense phagocytic activity leading to events as diverse as engulfment and destruction of extracellular material and the production of inflammatory mediators that may modulate both the immune and trophoblast invasiveness[60,61] (Figure 4).

The molecular and cellular contribution made by the above-mentioned extra-embryonic membranes, *i.e.,* exocoelomic cavity, amnion, yolk sac and trophoblast to the intra-embryonic mesoderm, could be essential for embryo development and organogenesis. Moreover, these primitive extra-embryonic structures can be internalized by the embryo at early development stages[50]. Consequently, the hypothesized re-expression of these extra-embryonic functions after injury during postnatal life could be a key process needed to repair the injured organism[2,41]. If so, the recapitulation of extra-embryonic functions through the organism could be internalized into the injured interstitium, thus inducing a process similar to the early embryonic process for tissue repair by regeneration and/or fibrosis.

INFLAMMATORY ENDOTHELIAL EGG

It could be proposed that recapitulation of extra-embryonic functions during wound repair is made up through the activation of two functional axes, namely: the coelomic-amniotic axis and the trophoblastic-vitelline axis. Both axes would polarize in the interstitium of the wounded tissue, thus promoting the development of a new tissue (Figure 4).

In surgical-related inflammation, the interstitium is surrounded by an inflamed heterogeneous endothelium. Thus, this inflammatory endothelium would get cellular and molecular mediators through the post-capillary venule endothelium, the high endothelial venule endothelium in the lymph nodes and, to a lesser degree, through the capillary endothelium. Ultimately, the lymphatic endothelium has a basic excretory function. The complex made up by this inflamed heterogeneous endothelium and the interstitial space of the injured tissue surrounded by it

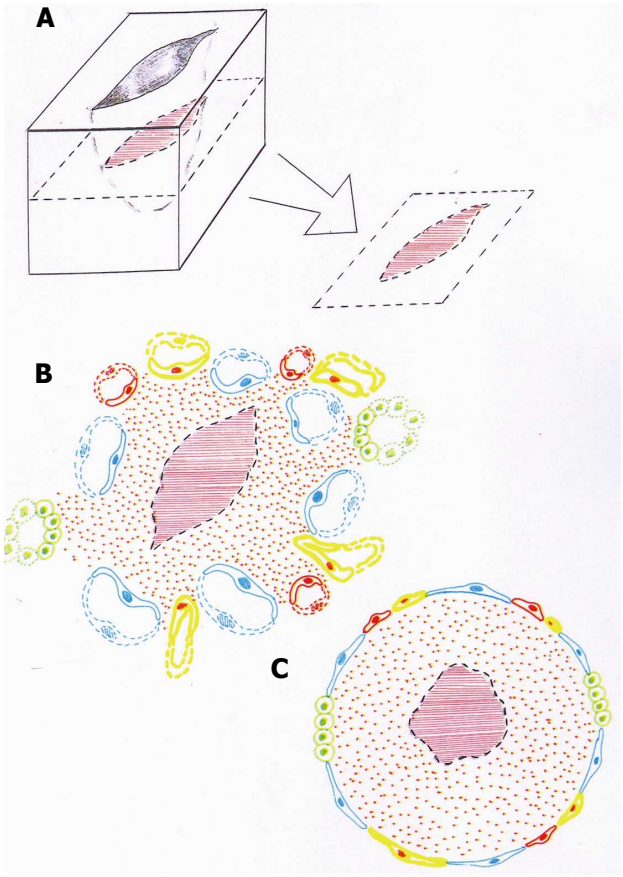

Figure 5 Figurative representation of a skin wound. The wound (A) is surrounded by different types of inflammatory venous, arterial and lymphatic endothelia (B). This heterogeneous inflammatory endothelium could be represented like a sheath of the inflamed interstitium that surrounds in turn the wound or broken tissue (C).

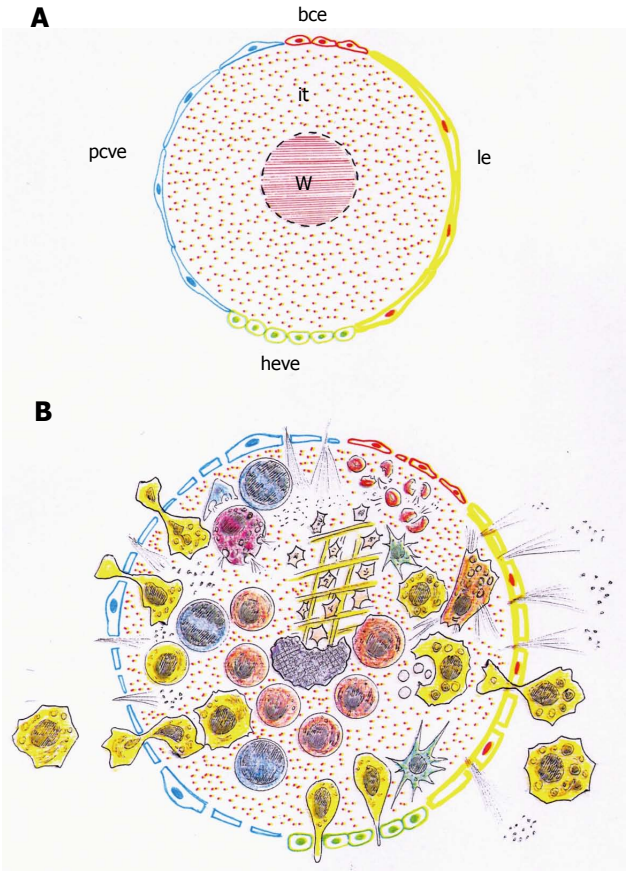

Figure 6 Schematic representations of the heterogeneous endothelium that surrounds the wounded tissue. A: The endothelium that cover the wound (W) and the damaged interstitium (it) are made up by the post-capillary venous endothelium (pcve), the high endothelial venular endothelium (heve), the lymphatic endothelium (le) and the blood capillary endothelium (bce); B: The inflammatory response is produced into the injured interstitium. The inflammatory mediators, molecules and cells, invade this interstitial space crossing through a sheath of heterogeneous endothelia.

has been compared with an "*endothelial egg*"[62] (Figures 5 and 6). Thus, in the interior of this heterogeneous endothelial sheath, the successive evolutive phases of wound repair with interstitial edema, activation of the lymphatic circulation and a hypoxic environment that could be an ideal stem cell niche, can be represented. Then, hemostasis by the formation of a platelet-fibrin clot occurs. After that, neutrophils, monocytes and lymphocytes are recruited and finally, new tissue is formed by regeneration, *i.e.*, keratinocytes and granulation tissue, *i.e.*, fibroblasts and endothelial cells, which form a substrate to complete the wound repair by fibrosis[12,14,30,34] (Figures 6 and 7).

However, cutaneous wound healing is not only a local process, but also a complex process involving systemic inflammatory alterations related to the stress response[2,62]. The magnitude of this systemic response may reflect the demands of the "*endothelial egg*" required for wound repair (Figure 7). In this sense, we have been trying to establish similarities between the complex pathophysiological mechanisms developed in wound healing and the pluripotential extra-embryonic pathways during embryonic development[2,10,41,62]. In this way, the recapitulation of coelomic-amniotic and trophoblast-vitelline functions is selectively integrated into the injured area. The recapitulation of the extra-embryonic coelomic and amniotic functions could be represented by initially activating the systemic neurogenic axis, while the latter recapitulation of the trophoblast and yolk sac functions would be carried out by activating the systemic bone-marrow axis (Figure 7).

RECAPITULATED COELOMIC-AMNIOTIC FUNCTIONS: A NEUROGENIC SYSTEMIC RESPONSE

The pathological neuromuscular response secondary to a wound induces sensory changes (stress, inflammatory pain, analgesia) and motor alterations (fight-to-flight and withdrawal reflexes, tachycardia and vasoconstriction-vasodilation). This upregulated extra-embryonic phenotype would induce a sudden and early neurogenic response with systemic cardiovascular, hemodynamic and hydroelectrolytic alterations[2,62]. Systemic and local ischemia-reperfusion produce sudden hydroelectrolytic changes associated with abnormal ion transport[63]. In this early response, cells that produce substances for export first

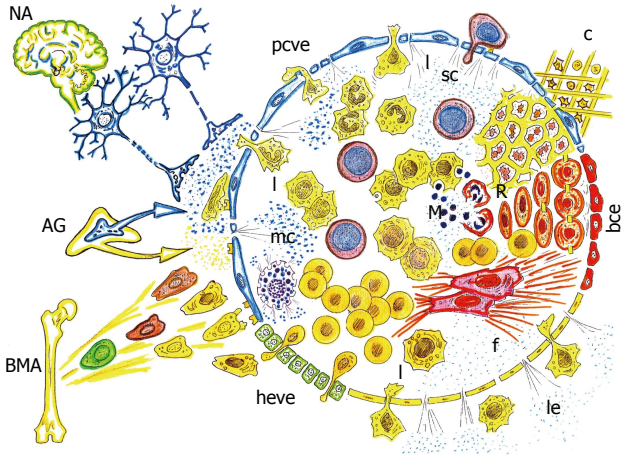

Figure 7 Neurogenic and bone marrow-related axes coupled in the inflamed endothelial egg, after wound. The upregulated extra-embryonic functions, *i.e.*, coelomic-amniotic or neurogenic, and trophoblastic-vitelline or bone marrow-related, are focused in the endothelial inflammatory egg, favoring the induction of a gastrulation-like phenotype, which evolves towards re-epithelization and fibrosis (scar) in post-natal life. NA: Neurogenic axis; AG: Adrenal gland; BMA: Bone marrow-related Axis; c: Coagulation; sc: Stem cell; mc: Mast cell. R: Regeneration; f: Fibrosis; l: Leukocytes; M: Microbiome. ve: Post-capillary venous endothelium; heve: High endothelial venular endothelium; le: Lymphatic endothelium; bce: Blood capillary endotelium; pcve: Psot capillary venous endothelium.

synthesize and then store large amounts of molecules, such as biogenic amines and neuropeptides in secretory vesicles ready for rapid release[64]. In this early neurogenic response, the activation of the hypothalamic-pituitary-adrenocortical, sympathetic-adrenal medullary and renin-angiotensin-aldosterone axes occur, with the release of catecholamines, glucocorticoids and mineralocorticoids. Consequently, selective accumulation of these mediators in the "*endothelial inflammatory egg*" is produced because endothelial permeability is increased, especially in postcapillary venules[2,62] (Figure 7).

RECAPITULATED TROPHOBLASTIC-VITELLINE FUNCTIONS: A BONE-MARROW-RELATED RESPONSE

The inflammatory bone marrow-related response induced by wounds could be considered both a key and complementary arm of the systemic response to injury. The inflammatory activation of the bone marrow stem cell niche indicates the stimulation of hematopoietic stem cells and mesenchymal stem cells, both which are multipotent stem cells[65-67]. Hematopoietic stem cells are the progenitors of all blood and immune cells. Macrophages generated from hematopoietic stem cells are the dominant phagocytes at wound-healing sites. Profibrotic macrophages, in particular, are intimately involved in wound healing through the production of mediators that directly activate fibroblasts, including transforming growth factor-beta (TGF-β), PDGF and IGF-1[28]. Nevertheless, although macrophages are required for the initiation and maintenance of fibrosis, they are also involved in its suppression, resolution and reversal[28,68]. Therefore, macrophage activation is best considered as a continuous spectrum of phenotypic characteristics[69]. In this context, circulating endothelial cells have also proved to be an important marker of vascular remodeling associated with wound healing. Angiogenesis is needed during embryonic development and plays important roles in wound healing and tissue ischemia throughout postnatal life[62]. Although the major physiological role of circulating endothelial progenitor cells is to maintain vascular integrity, they can also participate in revascularization of ischemic wounded tissues[70].

Furthermore, the upregulated trophoblastic-vitelline phenotype could mediate the inflammatory response through a lipid metabolic switch linked to steroid and acute phase response protein synthesis, respectively[2]. This slower response would therefore be developed by steroidogenic cells that store very little steroid hormones, in which case a rapid steroidogenic response would require immediate synthesis of new steroids, such as cortisol. The increase of the acute phase protein synthesis, *i.e.,* innate immunity, by the gut-liver axis is linked with the acute phase response and follows the upregulation of pro-inflammatory cytokines and chemokines[2,41,62].

COUPLING THE RECAPITULATED EXTRA-EMBRYONIC AXES IN THE "*INFLAMMATORY ENDOTHELIAL EGG*"

The systemic recapitulated extra-embryonic axes, *i.e.*, coelomic-amniotic and trophoblastic-vitelline, are focused and coupled in the endothelial inflammatory egg. This interstitial integration of both pathological axes, *i.e.*, neurogenic and bone-marrow-related in the wounded tissue, could finally induce a gastrulation-like process[41] (Figures 6 and 7). Gastrulation, which involves the "*de novo*" formation of reparative tissue, is based on the recapitulation of the intra-embryonic mesenchyme formation process[41]. In essence, the integration of both extraembryonic-related phenotypes coelomic-amniotic and trophoblastic-vitelline by the multipotent mesenchymal stem/stromal cells[67,71,72] would support the functional and metabolic heterogeneity needed for successively modulating their injured microenvironment during embryo development[50]. Therefore, the interaction of extra-embryonic functional axes recapitulated after injury in the interstitium of the damaged tissue allows for the recapitulation of the mechanisms characteristic of gastrulation, subsequently forming a mesenchyme in the endothelial inflammatory egg similar to that present in the early development phases[2,62].

Therefore, the early post-injury induction of extra-embryonic mechanisms that favors the beginning of the repair process[1] is undermined throughout the evolution of the wound healing reaction. In this way, the tissue that initiates its development inside the hypothesized endothe-

lial egg seems to suffer an immunological injury from the host organism. This reaction, similar to what takes place in organ transplantation, *i.e.*, host-versus-graft reaction, would explain the involution of the newly formed tissue until constructing, in the long term, the devitalized scar tissue. The study of those factors that induce this switch in the host organism, by which it gives up its gestating role and adopts a rejection attitude against already newly formed tissue, would explain why some authors consider that, in order to achieve tissue repair, inflammation is not needed[11].

CONCLUSION

In the current review, the wound healing reaction is considered a systemic inflammatory response made up by upregulated extra-embryonic functions, *i.e.*, coelomic-amniotic and trophoblastic-vitelline. The confluence and overlapping of these functions produce an injured tissue that would adopt an egg-like configuration that is one mainly made up of two structures: a round interstitial space surrounded by a heterogeneous endothelium. Therefore, cellular and molecular mediators from the extra-embryonic functions recapitulated by the injured organisms would induce a gastrulation-like process in this inflammatory endothelial egg from which tissue repair is produced either by regeneration and/or fibrosis.

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REVIEW

Multiple sclerosis and the role of immune cells

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Abstract

Multiple sclerosis (MS) is a complex disease with many different immune cells involved in its pathogenesis, and in particular T cells as the most recognized cell type. Recently, the innate immune system has also been researched for its effect on the disease. Hence, cells of the immune system play vital roles in either ameliorating or exacerbating the disease. The genetic and environmental factors, as well as the etiology and pathogenesis are of utmost importance for the development of MS. An insight into the roles play by T cells, B cells, natural killer cells, and dendritic cells in MS and the animal model experimental autoimmune encephalomyelitis, will be presented. Understanding the mechanisms of action for current therapeutic modalities should help developing new therapeutic tools to treat this disease and other autoimmune diseases.

Key words: Multiple sclerosis; Experimental autoimmune encephalomyelitis; Chemokines; Chemokine receptors; Glatiramer acetate; Central nervous system; T cells; B cells; Natural killer cells; Dendritic cells

Core tip: The role played by various immune cells in either ameliorating or exacerbating multiple sclerosis is discussed.

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INTRODUCTION

Recent developments in multiple sclerosis (MS) research have been staggering, including numerous new therapies either approved or at the brink of approval, new oral therapies and leaps made in genetics. This article will focus on the role that immune cells play in MS, and their involvement in mediating the effects of drugs used to treat relapsing-remitting (RR) MS patients.

GENERAL FEATURES OF MULTIPLE SCLEROSIS

The basic pathology of MS was recognized almost 150 years ago by Charcot who described a disease caused by sclerotic plaques in the central nervous system (CNS) of those affected[1]. There were other reports describing the pathology before this time, but failed to recognize it as a distinct disease[2]. It is reasonable to believe that MS has been prevalent also before these descriptions, as the disease often arises early in life[3]. Today, we know that MS is a chronic inflammatory, autoimmune, demyelinating and degenerative disease of the CNS[4]. Substantial discoveries have been made regarding its therapeutics, pathogenesis and genetics. Yet, there is no cure and no recognized definite cause, be it genetic, microbial or environmental. Although no definite cause of MS has yet been identified, substantial amounts of research point to a dual multifactorial influence of both genetics and environmental elements contributing to the development of the disease[5,6].

Genetic factors

The genetic component was first described after family studies were performed, with a family recurrence rate of about 20%-33%, and about 10-12 fold risk increase in first degree and three folds in second degree relatives[7,8]. For siblings or children of patients with MS, the overall risk was estimated to be 3%-5%[9], but twin studies show a higher risk (25%) if the sibling is a monozygotic twin[10,11].

The first risk allele to be identified was the human leukocyte antigen (HLA) class Ⅱ haplotype HLA-DRB 1501 in the early 1970's[12], which is also the allele with the strongest association with the disease[13]. The road to discovering additional alleles was complicated and hampered by insufficient methods[4,14]. In recent years large genome wide association studies (GWAS) using single nucleotide polymorphisms and comparing diseased to healthy individuals have made it possible to identify numerous alleles associated with MS with sufficient power and significance, most of these with low to modest MS risk association[5,15]. Researchers with the help from a huge GWAS consortium[13] identified over 50 susceptible loci, many of which are associated with the immune system, including genes encoding receptors for interleukin (IL)-2 and IL-7[15]. Other genes important for cytokine pathways include CXCR5, IL-12A, IL-12β, IL-12Rβ1 among others associated with the cytokine tumor necrosis factor α, as well as genes associated with co-stimulatory molecules such as CD80, CD86, CD37 were also described. GWAS studies have even identified risk association with genes that are important for current and new MS therapies including IL-2Rα (daclizumab) and VCAM1 (natalizumab), as well as genes related to environmental risk factor vitamin D_3[13]. Such knowledge while adding new information supports much of what is known about MS pathogenesis. Still, further research into these risk alleles is needed to improve our understanding of their associations with the disease.

Environmental factors

While family studies support a genetic association, they also show that genetics alone are not enough to develop MS, as shown by homozygous twins not both acquiring disease, even though one of them is diagnosed with MS. Other information supporting environmental effects include the geographic increase of MS with increasing latitude[16], and how individuals migrating from low-risk to high-risk areas acquire higher susceptibility if the migration occurs during childhood. Hence, the incidence of developing MS correlates with the risk in areas of childhood residence[4]. Additionally, studies have shown risk association during months of birth as spring births have higher risk of MS than autumn births[17].

Several environmental factors have been investigated. One hypothesis is that vitamin D_3 deficiency increases the risk of MS, as increased latitude is also correlated with lower blood vitamin D_3 levels. For instance, ecological studies showed the amount of exposure to sunlight was inversely correlated with the risk of MS, both by regional distribution and association with altitude, as well as by individual exposure to sunlight[18]. Sunlight is the main source of human vitamin D_3 through conversion of 7-dehydrocholesterol to previtamin D_3 in the skin, and through further metabolic steps to active hormone 1,25-Dihydroxyvitamin D_3[19]. It was also shown how vitamin D_3 intake may reduce the risk of MS because of latitude dependent deficiency, for instance in communities which consume higher amounts of vitamin D_3 rich fish[20]. These studies also point out the difficulties of such concepts, as confounding factors may be quite prevalent. There is also association with the experimental autoimmune encephalomyelitis (EAE) model, an animal model for MS, as dosing of 1,25(OH)2D_3 prevented the disease[21,22]. These effects may be induced by vitamin D_3 on the adaptive immune system not yet fully understood. Definite effects of supplementing patients with vitamin D_3 have not yet been shown, but some studies indicate that serum concentrations of vitamin D_3 may affect disease severity[19]. However, this field is quickly developing, and is being investigated for possible future prospects, for instance in preventing MS[23].

Another risk factor with strong association to MS is Epstein Barr virus (EBV) infection[24,25]. A major finding was that individuals who were seronegative for EBV had very low, almost no risk of acquiring MS when compared to seropositive individuals[24]. However, those patients who at some point infected with EBV are not necessarily at risk of developing MS[6]. Further, there was an increased risk of MS if a history of infectious mononucleosis and a temporal increase of EBV antibodies serum titers were present[26], and that MS patients were more often infected with EBV at later ages when compared to controls[27]. It has been hypothesized that EBV may mimic myelin basic protein (MBP) pathogenic antigens by presentation on HLA-DRB1*1501, hence, providing links to both environmental and genetic risk factors[28]. Although the association with MS is well investigated, the role of EBV in its pathology remains uncertain. However, EBV infection and mononucleosis as a priming or initiating factors for developing MS are seemingly likely[24]. Finally, many other factors have shown association with increased MS risk but are in need of further research to be conclusive. These include cigarette smoking, a diet rich in saturated but low in polyunsaturated fats, sex hormones, and socioeconomic status, among others[18,29]. Viruses other than EBV have also been implicated in the etiology of MS[30].

CLINICAL OBSERVATIONS

Natural history

Patients with MS show a wide variety of symptoms caused by lesions in the CNS affecting motor, sensory, visual or even autonomic functions. Lesions appear mainly in the white matter, but also appear in the grey matter. Hence, symptoms show great heterogeneity in both inter- and intra-individually ranging from slight tingling in the fingers to extreme fatigue or complete monocular loss of sight, depending on the lesion(s) localization[4]. Com-

monly, the patients present with a clinically isolated syndrome (CIS), followed by serial sub-acute relapses with varying time intervals. The number of annual relapses and time intervals between them varies among patients, but more than 1.5 relapses/year is a rare occasion[4]. Not all patients have subsequent disease activity after a single CIS, but risk of another episode is increased if white matter lesions are detected by MRI[31]. Between relapses the patients revert spontaneously to normal or near normal neurological function. This clinical subtype of MS is referred to as RR MS, and most patients are present with this form of MS[30]. The clinical course may later convert to a more progressive stage in about 65% of the patients, with fewer or no relapse activity and increasing irreversible disability. Patients have a mean age of about 40 years when they enter this stage, referred to as secondary progressive (SP) MS[32,33]. A minority of patients (about 10%-15%) may present with progressive disability and no relapsing activity, this is called primary progressive (PP) MS[34]. This subtype usually has a later onset than RRMS, at a mean age of around 40 years[33].

Patients with MS experience decades of disease and eventual progression. Due to many complications which occur alongside increased disability, the average life expectancy is reduced by about 5-10 years, and has a median time of 30 years between onset and death[35]. It remains to be seen whether current or future therapeutic methods may extend the time until irreversible disability occurs.

ROLE OF IMMUNE CELLS IN MS

The blood brain barrier

An important finding in MS lesion is the disruption of the blood brain barrier (BBB), and hence a basic understanding of this barrier is essential to understand the pathogenesis of the disease[36]. The BBB is a functional and anatomical barrier separating the blood from neurons in the CNS. It consists of both the vascular wall, CNS astrocytes covering these with glia limitans, and the perivascular space in between[37]. The BBB has several important functions vital for the brain to function properly, including maintenance of proper ionic concentrations through dynamic regulation. The BBB may be viewed as a concept rather than an actual barrier. Even though the word barrier has a certain static ring to it, the BBB is rather dynamic allowing for instance immunological surveillance[38,39]. Lesions occur when leukocytes migrate into the brain and inflammation ensues. This is a two-step process, involving an initial migration across post-capillary venules into perivascular space, and further migration through the glia limitans into the brain parenchyma[37]. The perivascular space allows normal immunosurveillance by monocytes, as this space also serves as the brains' lymphatic drainage[37]. There are at least two additional routes for leukocytes to enter the CNS, as shown by accumulation of immune cells at these sites in animal models, which include the blood-cerebrospinal fluid (CSF) barrier in the choroid plexus, and through meningeal arteries[39].

Role of T cells

The role of T cells has always been considered central in MS pathogenesis, much due to the experimental animal model EAE, but also due to the strong genetic association with HLA class Ⅱ genes[5]. EAE is a widely used animal model for MS, and is induced by immunizing mice or other rodents with myelin peptides, or by adoptively transferring myelin-reactive T-cells[6]. Hence, they cause a T cell mediated acute autoimmune reaction against myelin in the rodents CNS, with signs and symptoms that are similar to those seen in MS. The cellular pathogenesis of MS is however, more complicated than this[40]. For a long time auto-reactive $CD4^+$ T cells secreting interferon-gamma (IFN-γ), were thought to be the main mediators of the inflammation causing MS lesions[4]. Further research suggests numerous other cell types and cell subsets are also involved, with key roles assigned to T helper 17 (Th17) cells[41]. These T cells secrete the pro-inflammatory cytokines IL-17, IL-6 and are regulated by IL-23[42,43]. It is commonly believed that disease occurs when these inflammatory cells and/or other cell types become deregulated and a transition from physiological surveillance to a pathological immune response occurs[4,30].

Since EAE is considered a model of acute inflammation, it has been used to explore what cells are important for this process. It was shown in mice with EAE that $CD4^+$ Th17 cells are necessary to develop EAE[44]. Studies of lesions from MS patients confirmed an overwhelming presence of $CD4^+$ cells secreting IL-17 in active lesions. However, in MS lesions both $CD4^+$ and $CD8^+$ cells express IL-17[45]. Chemokine receptor CCR6 expressed on Th17 cells facilitates transport through the choroid plexus into the CSF and perivascular space by interacting with CCL20/MIP-3α expressed on endothelium[46]. Th17 cells may also produce GM-CSF promoted by resident antigen presenting cells (APCs) secreting IL-23, which then initiates a positive feedback loop as the same APCs are stimulated by GM-CSF[30,47,48]. Th17 cells may further increase permeability of the BBB by disrupting the endothelial tight junctions due to the secretion of IL-17 and IL-22, and through interactions with endothelium allowing further attraction of $CD4^+$ subsets as well as other immune cells. Consequently, initiating pathological cascade of inflammation, perivascular infiltrates and damage to neurons and glia cells[30,45]. One identified damaging molecule is Granzyme B secreted by the same Th17 cells[49]. However, to gain access into the parenchyma, the cells must traverse the glia limitans. This is thought to be mediated by perivascular APCs and macrophages secreting matrix metalloproteinases (MMP) 2 and MMP-9 which are gelatinases able to cleave dystroglycan, a transmembrane receptor anchoring astrocytes end feet to the parenchymal basement membrane. When mice were knocked down for MMP-2 and MMP-9, they became resistant to EAE as T-cells became trapped in perivascular space[50]. In summary, this cascade may be viewed as a stepwise model, with an initial attraction and migration of Th17 cells into the CSF and perivascular spaces, and later increased permeability of the BBB al-

Table 1 Chemokines receptor expression on T cells and T cell clones isolated from MS patient

Receptor	Main role	Ligands	$CD4^+$ subsets			PBL-74		PBL-78		CSF-25		CSF-26	
			Th1	Th2	Naive	Unact	Act	Unact	Act	Unact	Act	Unact	Act
CXC family													
CXCR1	I	CXCL6, CXCL7, CXCL8											
CXCR2	I	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8											
CXCR3	I	CXCL9, CXCL10, CXCL11	H			H	M	H	M	H	M	H	L
CXCR4	C	CXCL12			H								
CXCR5	C	CXCL13		M									
CXCR6	I	CXCL16											
CXCR7	D	CXCL11, CXCL12											
CC family													
CCR1	I	CCL3, CCL5, CCL7, CCL8, CCL15, CCL16, CCL23	M										
CCR2	I	CCL2, CCL7, CCL8, CCL13, CCL16	H	H									
CCR3	I	CCL5 ,CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28		H									
CCR4	D	CCL17, CCL22		H		H	H	H	M	H	H	L	
CCR5	I	CCL3, CCL4, CCL5, CCL8, CCL11, CCL14, CCL16	H			H	M	L		H	M		
CCR6	D	CCL20				M		M		L			
CCR7	C	CCL19, CCL21			H								
CCR8	I	CCL1, CCL16		H									
CCR9	D	CCL25,											
CCR10	I	CCL27, CCL28						L	L				
Other													
XCR1	I	XCL1, XCL2											
CX3CR1	D	CX3CL1											

Data adapted from Høglund *et al*[53], Maghazachi[90], Zlotnik *et al*[117] and Sallusto et al[118] . I: Inflammatory; C: Constitutive; D: Dual activities; Unact: Unactivated; Act: Activated; PBL: Peripheral blood lymphocytes; CSF: Cerebrospinal fluid. H: High expression; M: Medium expression; L: Low expression.

lowing additional inflammatory cells access, and later a complete disruption of the BBB with damage to the CNS occurs, resulting in active lesions and potential clinical exacerbation of the disease[25,30].

Although our understanding of how inflammation may be initiated has increased with the advent of the Th17 hypothesis, much remains to be discovered of how T cells and other cell types are recruited into the CNS during inflammation. It is known that chemokines are important for recruiting these cells across the BBB. Chemokines are classified into subfamilies, consisting of CXC, CC, C and CX3C. While some are constitutively expressed, others are up-regulated during inflammation[51]. In MS lesions, chemokines expressed on post-capillary venule endothelial walls and bind chemokine receptors expressed on T cells, allowing extravasation. Chemokine concentration gradients in tissues allow the cells to be further guided towards the sites of inflammation[52].

We explored the role of chemokine receptors expression, which include CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR9, CCR10, CXCR1, CXCR3, CXCR4, CXCR5 and CXCR6 on four T cell clones isolated from the blood or CSF of MS patient who was treated with the drug glatiramer acetate (GA). We observed that only four chemokine receptors warranted further study. These were CCR4, CCR5, CCR6 and CXCR3[53]. The fact that all four clones from both peripheral blood and CSF expressed similar patterns of chemokine receptors could be due to migration of the same clones from blood into the CSF, plausibly using CCR5 or CCR6 for entry (Table 1). When these cells were activated, all chemokine receptors expression with the exception of CCR4 was markedly reduced. The migration pattern corresponded well with the expression of receptors observed, with reduced migration observed when receptors were reduced or no longer expressed. Similarly after activation, migration towards chemokines was less robust. These observations support the hypothesis of diminished effects of GA due to inflammation and premature activation of GA-reactive cells. As migration into the CNS is a necessary step of the bystander suppression hypothesis[24], knowledge of how these cells migrate into the CNS is important in order to possibly enhance the effectiveness of this drug.

These results could in part explain why aggressive immunosuppression followed by maintenance therapy is very effective. Such combination therapy is relatively new to MS treatment, but has been used in other autoimmune diseases. Inspired by this, researchers applied combination therapies which resulted in a better outcome for RRMS patients. Several combinations have been tested, some show more promise than others. It was for instance described how patients treated with a short induction of mitoxantrone followed by longer term GA therapy showed a reduction in new relapses compared to GA alone, as well promising results regarding gadolinium enhanced lesion load[54-57]. This suggests possible synergistic effects among these drugs. Mitoxantrone is a powerful immunosuppres-

sive drug that is approved for treatment of RRMS and secondary progressive MS (SPMS) in the United States. By suppressing inflammation induced by autoreactive T cells such as Th17 and/or Th1, this drug may pave the way for GA-reactive cell migration into the CNS upon reconstitution of the immune system. This way, the cells may mediate their bystander suppression without being impeded by an inflammatory environment. However, while initial studies of this showed important clinical efficacy, further studies are warranted in order to determine whether this treatment regimen is better suited than newer therapies such as alemtuzumab and other future immunomodulators.

The presence of inflammatory T cells in perivascular space and parenchyma triggers recruitment of more T cells, as well as B cells, dendritic cells, microglia and natural killer (NK) cells. The cytokines secreted may by themselves cause damage to the surrounding tissues[58]. Complement depositions, opsonization and local activation of microglia and macrophages causing demyelination[59], and neuronal cell death are few examples of the effects of cytokines[60]. Axonal damage is also present[61,62], and the degree of this was associated with abundance of microglia and $CD8^+$ T cells[63]. Clonally expanded $CD8^+$ T cells are present usually at the lesion edge, as well as in the perivascular infiltrates, indicating a specific antigen response and also possibly responsible for damages to neurons through cell dependent cytotoxicity[64-67]. It was also demonstrated that clonal γδ T cells are present in MS lesions[68]. When the lesion acute phase is over, removal of cellular debris starts, and simultaneously remyelination occurs within the MS plaque. A minority of the patients (about 20%) show more abundant remyelination[69].

In addition, a study showed that MS patients had a significant loss of effector function in the regulatory $CD4^+CD25^+$ subset of T cells (Treg), when compared to healthy individuals[70]. Another study showed reduced frequency of this subset in general as well as reduced expression of transcription factor FOXP3[71]. Although circulating numbers may be the same in patients and healthy individuals, the suppressive potential of Treg cells in MS patients was reduced[72]. Interestingly, these T cells were also able to produce IL-17 when stimulated with APCs in the presence of IL-2 and IL-15[73], but retained their suppressive abilities dependent on the surrounding cytokine environment[74]. Although this regulatory subset may inhibit auto reactive T cells, it is not certain where such inhibition may occur[41].

Finally, it may be that MS is not initially caused by the adaptive immune system at all, but rather as a response to an intrinsic CNS neurodegeneration, indicating that inflammation may follow initial axonal degeneration caused by a currently unknown factor. This is supported by the fact that progression of disease is mainly caused by the amount of lost neurons[30]. In addition, there seems to be a correlation between age at onset than initial clinical course on the disease progression[33].

Role of B cells

The most obvious argument for B cells being involved in MS pathogenesis is the presence of immunoglobulins such as immunoglobulin G1 in the CSF, as detected by isoelectric focusing or gel electrophoresis in as many as 95% of diagnosed patients. Although myelin reactive antibodies have been detected, their relevance is not certain[25], much like the role of antigens. In MS lesions, there have been findings of both immunoglobulin and complement which may suggest a pathogenic role[75,76]. Additionally, B lymphoid follicles, T cells and APCs were identified in the meninges of patients at later stages of MS (SPMS but not PPMS)[76,77]. B cells may contribute to the pathology through antigen presentation, cell interactions or production of immunoglobulins from plasma cells, although B cell activity may represent a response to the autoimmune reaction, rather than to a primary inducer[25].

In EAE, the antigens responsible for the disease have been described. By immunizing the animal with myelin proteins such as MBP, proteolipid protein or myelin oligodendrocyte glycoprotein (MOG), disease can be induced[78]. In humans, such common antigens remain unknown in spite of several attempts to identify them[25,30]. Several candidate antigens are being or have been investigated, but no single candidate has been targeted as the one responsible. Myelin or peptides derived from myelin were thought to be good candidates due to the similarity between EAE and MS, but the responses to these antigens have proven to be unspecific and may suggest that several antigens are involved and/or an extensive epitope spreading occurring after initiation of the disease[30,41,79]. One recently suggested antigen is αβ-crystallin, which contrary to previous candidates is not present in human myelin, but rather is detected in early active MS lesions, and patients have antibodies against it in their CSF[6]. When the gene encoding αβ-crystallin was knocked down in mice, a more intense inflammatory EAE with higher cytokine load occurred[80]. This suggests a protective role that may be disrupted by a pathogenic immune response. Another candidate not directly associated with myelin is neurofascin, expressed on neuronal axons. As antibodies have been detected in patients with MS, this may contribute to axonal damage[81].

Role of NK cells

NK cells are large granular lymphocytes that possess the ability to spontaneously lyse target cells without a prior sensitization[82]. NK cells also have immunoregulatory features, including secretion of cytokines, chemokines and cell to cell contact[83,84]. Functionally, these cells are important in immune responses to viral infections as well as controlling tumor growths[85,86]. The activities of these cells are regulated by activating and inhibitory receptors, which by intracellular integration of challenges and inhibitions determine the cell course of action[87]. NK cells recognize and are activated by cells that are in distress by detecting stress induced ligands on target cells through natural cytotoxicity receptors, such as NKp30, NKp44, or NKp46, and the C-type lectin receptor NKG2D, among others[87,88]. In healthy cells however, activating

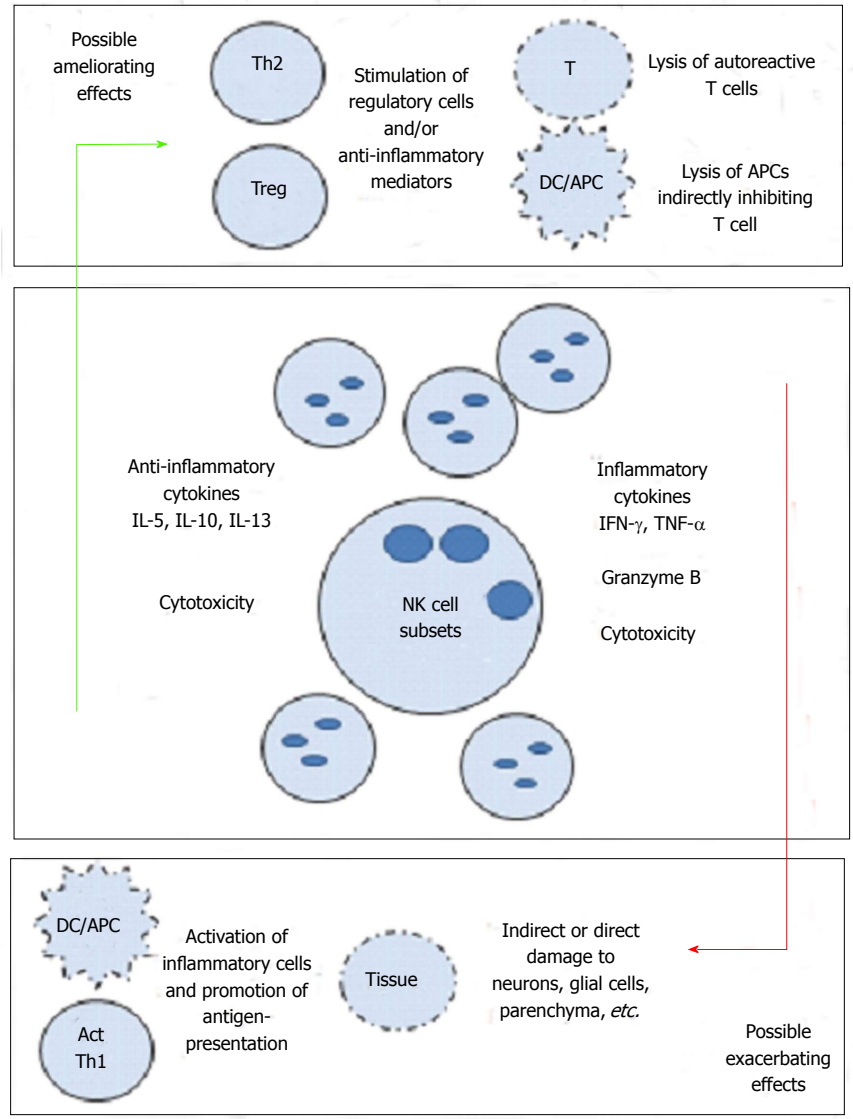

Figure 1 Natural killer cells influence multiple sclerosis pathogenesis in both protective and exacerbating ways. Lysis of either auto-reactive cells or antigen presenting cells (APCs) may protect the central nervous system (CNS) from damage. Stimulation of protective Th2 or Treg cells may also encourage an anti-inflammatory environment. On the other hand, stimulation of APCs or auto-reactive cells may have opposite effects. IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon; NK: Natural killer.

factors are held in equilibrium by inhibiting signals. NK cells express numerous receptors that inhibit activation, including members of the killer-cell immunoglobulin-like receptor (KIR) family that interact with HLA-I molecules and CD94-NKG2A that interact with HLA-E. In the absence of these "self" ligands, NK cells are activated and consequently, kill target cells[89].

In human blood, NK cells constitute about 2% to 18% of the total circulating lymphocytes[90,91], and can be classified into subsets based on expression of surface receptors. $CD16^{+}CD56^{-}$ constitute about 85%-90% of circulating NK cells, are highly cytotoxic but produce little cytokines, while $CD16^{-}CD56^{+}$ cells are less cytotoxic but effective cytokine producers[92]. NK cells may also be classified based on their cytokine expression. Similar to Th1/Th2 subsets, these cells were divided into NK1 expressing IFN-γ, or NK2 expressing IL-5 and IL-13[93]. Further research divided these NK cells into even smaller subsets, recognized as NK22 secreting IL-22 and found in lymphoid tissues of the gastrointestinal tract[94], or NK17/NK1 cells secreting IL-17 and IFN-γ when stimulated with IL-2[95].

The role of NK cells in autoimmune diseases is being investigated, and is highly debated as both positive and negative associations have been observed[96-99]. In the EAE model, depletion of NK cells resulted in a severe relapsing EAE, and more pronounced CNS pathology[100-103]. This suggests protective effects for NK cells, especially since the depletion was associated with increased $CD4^{+}$ T cell activity and hence, may be associated with direct killing of these cells[88,104]. Also in EAE, mice deficient in the chemokine receptor CX_3CR1 had more severe EAE. This receptor is necessary for the recruitment of NK cells into the CNS, providing evidence showing that NK cell infiltrating into the CNS represents an important event in ameliorating and controlling the disease[105]. In the same mice there were increased responses from Th17 cells locally in CNS, suggesting

a plausible role for NK cells in curbing these cells[106]. Conflicting with these results, it was found that depletion of NK cells in MOG-induced EAE actually ameliorated the disease[107]. Additionally, by stimulating NK cells to produce IFN-γ, these cells may also cause inflammation in part by stimulating Th1 cell response[108,109]. These findings represent detrimental effects of NK cells eliciting inflammatory lesions and exacerbating the inflammatory response. Much of the confusion regarding conflicting findings may be attributed to failure to recognize different effects by different subsets, which should encourage further investigation into this field.

The functional activity of NK cells is variable and is generally lower in MS patients than in healthy individuals[110]. Periods of reduced NK cell numbers in the blood was associated with a higher relapse tendency after. The same study also found a correlation between high mean NK cell activity and total lesion load determined by MRI[111]. As reduced numbers of NK cells were thought to be mediated by migration into tissues including the CNS, this could indicate a pathological role for NK cells. However, it could also be viewed as a risk factor for new attacks due to reduced activity of NK cells. In a more recent study, researchers found a reduced number of $CD8^{low}CD56^{+}CD3^{-}CD4^{-}$ cells in untreated patients with CIS, when compared to healthy controls[112].

Along these associations, there have been speculations as to how exactly NK cells mediate their effects, be it positive or negative. One suggested pathway was interactions between NK cells and dendritic cells[97,101]. The cognate and non-cognate interactions among NK cells and DCs have been previously described. Activated NK cells are also able to kill immature (i) DCs, but mature (m) DCs are spared. How and where these cells interact are not clear, but it was hypothesized that such interactions may occur in inflamed tissues. Our work on the effects of GA on NK cells in MS patients and healthy individuals shows that GA influences this cross-talk between DCs and NK cells. Consequently, we hypothesized that this may impede antigen presentation to auto reactive T cells (Th17 or Th1), and may further reduce inflammation. We described that NK cells stimulated *in vitro* with GA became more cytotoxic towards autologous and allogeneic iDCs and mDCs[113]. Furthermore, we described how NK cells from EAE mice treated with GA were more cytotoxic than NK cells isolated from EAE mice treated with vehicle alone, which was associated with ameliorating the disease[114]. While these findings support a mechanism of action for GA on NK cells, it had to be further addressed in humans with in vivo exposure to the drug. Consequently, we examined the activities of NK cells and DCs in MS patients receiving the drug GA for one year. In this study, we compared the cytotoxicity levels before treatment with those observed after treatment. We demonstrated that NK cells isolated from GA-dosed MS patients had significantly increased cytotoxicity against K562 tumor cells. Trends of significantly increased cytotoxicity were also observed against both iDC and mDC in the same patients[115]. In summary, it seems that GA increases the cytotoxic activity of NK cells when compared to pretreatment levels. The increased cytotoxicity correlated well with the elevated expression of activating NK cells cytotoxicity receptors. Figure 1 represents our current knowledge regarding the role that NK cells might play in MS.

CONCLUSION

The evidence for a complex immunopathology has become ever clearer with the reveal of numerous genes associated with the immune system carrying risk of developing MS, novel cell subsets being discovered and shown to possibly be related to the pathogenesis, and knowledge of how disease modifying therapies (DMT) target several immune cells rather than single subsets. All of these call for continuous bedside-to-bench research into how the newer DMTs enhance, inhibit or modulate the immune system of patients resulting in a better clinical course than without such treatment. The introduction of the new oral therapeutics teriflunomide (Aubagio®), fingolimod (Gilenya®) and dimethyl fumarate (Tecfidera®), all with incomplete understandings of their mechanisms of action on the immune system, will be particularly interesting to follow in this regard.

So far, research into how immune cells act in MS patients, EAE models and *in vitro* have primarily focused on single subsets or categorically on either adaptive- or innate immune cells. Recent knowledge of how NK cells may belong in a grey area between these traditional groupings challenges this concept[116]. For instance, while our own research focused on the interactions between NK and dendritic cells, similar interactions between NK and B cells would be of interest, as B cells also have antigen presenting abilities. NK cells also have the ability to modify or lyse T cells, and hence investigations into how these cells interact in MS patients or in the EAE model could improve our understanding of the regulation or dysregulation the immune systems of MS patients.

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OBSERVATIONAL STUDY

Oxidative stress and labile plasmatic iron in anemic patients following blood therapy

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Abstract

AIM: To determine the plasmatic iron content and evaluate the oxidative stress (OS) markers in subjects receiving blood therapy.

METHODS: Thirty-nine individuals with unspecified anemia receiving blood transfusions and 15 healthy subjects were included in the study. Anemic subjects were divided into three subgrouP: (1) those that received up to five blood transfusions (n = 14); (2) those that received from five to ten transfusions (n = 11); and (3) those that received more than ten transfusions (n = 14). Blood samples were collected by venous arm puncture and stored in tubes containing heparin. The plasma and cells were separated by centrifugation and subsequently used for analyses. Statistical analyses were performed using Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison tests when appropriate.

RESULTS: The eletrophoretic hemoglobin profiles of the subjects included in this study indicated that no patients presented with hemoglobinopathy. Labile plasmatic iron, ferritin, protein carbonyl, thiobarbituric acid-reactive substances (TBARS) and dichlorofluorescein diacetate oxidation were significantly higher ($P < 0.05$), whereas total thiol levels were significantly lower ($P < 0.05$) in transfused subjects compared to controls. Additionally, the activity of catalase, superoxide dismutase and glutathione peroxidase were significantly lower in the transfused subjects ($P < 0.05$). Antioxidant enzyme activities and total thiol levels were positively correlated ($P < 0.05$), and negatively correlated with the levels of protein carbonyl and TBARS ($P < 0.05$). In contrast, protein carbonyl and TBARS were positively correlated ($P < 0.05$). Altogether, these data confirm the involvement of OS in patients following therapy with repeated blood transfusions.

CONCLUSION: Our data reveal that changes in OS markers are correlated with levels of labile plasmatic iron and ferritin and the number of transfusions.

Key words: Antioxidant enzymes; Labile iron content;

Oxidative stress; Polytransfused subjects

Core tip: Here, the readers will find important information regarding iron accumulation and its correlation with oxidative damage markers in anemic subjects following blood therapy. This research, regarding iron accumulation and its associated toxicology is remarkable because the mechanism(s) involved in its mode of action are not fully understood. Thus, our data are extremely important for research concerning the involvement of iron overload on the development of human diseases.

Fernandes MS, Rissi TT, Zuravski L, Mezzomo J, Vargas CR, Folmer V, Soares FAA, Manfredini V, Ahmed M, Puntel RL. Oxidative stress and labile plasmatic iron in anemic patients following blood therapy. *World J Exp Med* 2014; 4(3): 38-45 Available from: URL: http://www.wjgnet.com/2220-315X/full/v4/i3/38.htm DOI: http://dx.doi.org/10.5493/wjem.v4.i3.38

INTRODUCTION

Iron is an essential element of cells that participates in various cellular processes due to its ability to accept and donate electrons, interconverting between Fe^{3+} and Fe^{2+} forms[1]. However, this redox property renders iron potentially toxic in biologic systems. The labile plasmatic iron (LPI) component of non-transferrin-bound iron is redox-active, chelatable and capable of permeating into organs to induce tissue iron overload[2]. Thus, LPI is an accessible diagnostic marker of iron overload and cell toxicity[2]. Moreover, LPI can participate in the Fenton reaction and generate a large amount of reactive oxygen species (ROS)[3]. To prevent ROS overproduction, circulating and intracellular free iron are tightly regulated by binding to transferrin, ferritin and other proteins[4,5]. However, the iron balance can be disrupted in some situations, such as with chronic anemia, repeated blood transfusions, and following increased gastrointestinal absorption, which lead to iron overload[6]. Therefore, subjects undergoing repeated blood transfusions are at risk of iron-associated toxicity[7].

Elevated tissue iron can overwhelm protective mechanisms and lead to an increase in iron complexes with small molecules, such as nucleotides and citrate, in the serum of transfusion patients and also within cytoplasm and organelles[8,9]. Furthermore, repeated blood transfusions increase the levels of iron available to generate catalytically active complexes, free radicals and oxidative damage[9]. Thus, LPI promotes free radical formation that culminates in the oxidation of biomolecules. Accordingly, iron overload in humans and in experimental animals is associated with oxidative stress (OS)[10]. Indeed, it is known that an imbalance in the oxidant/antioxidant status of the cell is associated with OS, leading to important cellular macromolecule modifications and cell damage[11]. The cell injury observed in patients with iron overload is attributed to OS[12]. Hence, the oxidation reactions result in the formation of lipid peroxides and protein carbonyls, damaged deoxyribonucleic acid bases, and mitochondrial dysfunction[13]. Additionally, individuals with an iron overload demonstrate impaired antioxidant defenses[6]. Accordingly, the long-term consequence of chronic iron overload is organ injury, which could contribute to the initiation and development of several metabolic disorders, such as endocrinopathies, diabetes mellitus, cirrhosis, hypogonadism and heart failure[14].

In general, oxidative damage of biomolecules can be counteracted by enzymatic as well as non-enzymatic defenses. Indeed, humans have several biologic mechanisms to defend against intracellular OS. One of the most important mechanisms involves the actions of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)[15]. In spite of a well-developed antioxidant defense system, cells can still be oxidatively damaged under some pathologic conditions[11].

Data concerning labile iron accumulation in anemic subjects receiving repeated blood transfusions and the association with oxidative damage markers are scarce in the literature. We hypothesize that OS correlates with LPI in anemic patients following therapy with repeated blood transfusions. In this study, we evaluated OS markers and the activity of enzymatic antioxidant defenses in the blood of patients receiving repeated transfusions and in control subjects (not transfused). Additionally, we determined the LPI and ferritin levels in these subjects and correlated both parameters with other evaluated markers.

MATERIALS AND METHODS

Chemicals

1,1,3,3-tetramethoxypropane, 2-thiobarbituric acid, sodium dodecyl sulfate, 5,5'-dithiobis-(2-nitrobenzoic acid), trichloroacetic acid, 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), and 2,4-dinitrophenylhydrazine, were purchased from Sigma (St. Louis, MO, United States). The kit for iron determination was obtained from Bio-Systems Corp. (Beloit, WI, United States), kits for measuring SOD (RANSOD) and GPx (RANSEL) were purchased from Randox Laboratories Ltf. (Crumlin, United Kingdom), and the Total Protein kit for protein determination was obtained from BioClin (Delft, Netherlands). All the other chemicals were commercial products of the highest purity grade available.

Subjects

This study was approved by the Ethics Committee in Research of Universidade Federal do Pampa. Altogether, 39 individuals with unspecified anemia receiving blood transfusions and 15 healthy subjects (blood donors) from the Banco de Sangue do Municipio de Uruguaiana were included in the study. Since most of our patients were male, the female patients were excluded from this study. Thus, both anemic and control healthy individuals were male. Anemic individuals were included in the study if they were diagnosed according to the International

Classification of Diseases (anemia unspecified, ICD 10: D64.9), and were not diagnosed with other diseases, such as cancer, renal failure, hepatic disease, blood loss or others. Additionally, anemic patients had received blood therapy during the year prior to collection (*i.e.*, no more than 12 mo from the first transfusion until sample collection). Additionally, it is important to mention here that the sample collection was done before a new transfusion, namely clinical screening. The anemic subjects were divided into three subgrouP: (1) those that received less than five blood transfusions (n = 14); (2) those that received from five to ten blood transfusions (n = 11); and (3) those that received more than ten blood transfusions (n = 14).

Sample collection

Blood from controls and anemic subjects was collected by venous arm puncture and stored in tubes containing heparin. The plasma and cells were separated by centrifugation at 1500 r/min for 10 min and were subsequently used for biochemical analyses. All biochemical assays were done in duplicate or triplicate, depending on availability of samples.

Analysis of hemoglobin

The electrophoretic analysis of hemoglobin was performed using a Minicap system (Sebia, Norcross, France) according to the manufacturer's instructions, and controls were run with each test. The Minicap system uses the principle of capillary electrophoresis in free solution. Charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electro-osmotic flow. Electropherograms were expressed with zones divided from Z1 to Z15 based on standardizing the location of hemoglobin as previously described[16].

Measurement of LPI

LPI refers to non-heme bound, non-ferritin bound and non-transferrin-bound iron (*i.e.*, free iron) according to the previously validated convention[17]. The LPI content was determined by its reactivity with ferrozine, in the presence of the denaturant sodium dodecyl sulfate and the reducing agents ascorbate and sodium metabisulphite, as previously described[18,19]. The results are expressed as μg/dL.

Ferritin

Ferritin content was determined as described by Bernard and Lauwerys[20]. Serum ferritin causes agglutination of latex particles coated with anti-human ferritin that is proportional to the concentration of ferritin and can be measured by turbidimetry. The results are expressed as μg/L ferritin.

Protein carbonyl determination

Protein carbonyl content, which is indicative of oxidation, was determined as described by Levine *et al*[21]. Plasma samples were added to 0.2 mL of 10% trichloroacetic acid and placed on ice for 5 min. After centrifugation (5 min), samples were incubated for 90 min at 37 ℃ with 1 mL of 10 mmol/L 2,4-dinitrophenylhydrazine in 2 mol/L HCl. Finally, proteins were dissolved in 6 mol/L guanidine and interference was removed after washing with ethanol-ethyl acetate 1:1 (v/v). The extent of the damage was estimated by reading absorbance at 370 nm. The results are expressed as nmol carbonyl/mg protein.

Determination of thiobarbituric acid reactive substances levels

Levels of thiobarbituric acid reactive substances (TBARS) in plasma were determined using the method described by Ohkawa *et al*[22]. In brief, samples were incubated in acidic medium containing 0.45% sodium dodecyl sulfate and 0.6% thiobarbituric acid at 100°C for 60 min. After centrifugation, the reaction product was determined at 532 nm using a 1,1,3,3-tetramethoxypropaneas standard and the results are expressed as nmol malondialdehyde/mg protein.

Total thiol determination

Plasmatic total thiol was determined as described by Ellman *et al*[23]. The colorimetric assay was carried out in 1 mol/L phosphate buffer (pH 7.4) and calculated against a standard curve constructed with glutathione. Total thiol content is expressed as nmol total thiol/mg protein.

Determination of DCHF-DA oxidation

The determination of intracellular oxidant production was based on the cleavage of DCHF-DA to DCHF, which fluoresces when oxidized by ROS according to previously described methods[24]. The plasma sample was diluted (1:10) in 10 mmol/L Tris-HCl buffer. Then, 50 μL of diluted plasma was incubated with 10 μmol/L DCHF-DA at 37 ℃ for 20 min. The fluorescence emission at 520 nm was measured using a Perkin-Elmer spectrofluorometer with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The results are expressed as arbitrary fluorescence units.

CAT activity

CAT activity was measured by the method previously described[25]. Packed erythrocytes were hemolyzed by adding 100 volumes of distilled water, then 20 μL of this hemolyzed sample was added to a cuvette and the reaction was started by the addition of 100 μL of freshly prepared 300 mmol/L H_2O_2 in phosphate buffer (50 mmol/L, pH 7.0) to give a final volume of 1 mL. The rate of H_2O_2 decomposition was measured by a spectrophotometer at 240 nm for a duration of 2 min. The CAT activity is expressed as UI/mg protein.

SOD activity

SOD activity was measured in erythrocytes using a RANSOD kit, which uses xanthine and xanthine

Table 1 Subject characteristics

Characteristics	Controls (n = 15)	Transfusions		
		< 5 (n = 14)	5-10 (n = 11)	> 10 (n = 14)
Age (yr)	40.1 (20–50)	62.8 (24–92)	64.8 (49–84)	57.5 (24–74)
Number of transfusions	0 (0)	3.20 (2–4)	7.17 (5–9)	18.78 (14–26)
Hemoglobin (g/dL)	13.8 ± 0.5	7.5 ± 2.1	6.75 ± 0.5	4.9 ± 0.9
Labile iron content (μg/dL)	108.9 ± 13.8	149.2 ± 45.1	216.2 ± 68.3[a]	366.9 ± 68.5[a]
Ferritin (μg/L)	219.6 ± 18.2	190.3 ± 11.7	221.2 ± 16.1	277.5 ± 27.5[a]

Values are presented as median (range), or mean ± standard deviation; [a]P < 0.05 *vs* control.

oxidase to produce superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazol chloride to form formazan red. The SOD activity was measured by the degree of inhibition of this reaction at 505 nm and is expressed as UI/mg protein.

GPx activity

GPx activity was determined in erythrocytes using the RANSEL kit according to the method previously described[26]. The GPx activity is expressed as UI/mg protein.

Protein determination

The protein content was determined by the biuret method using the Total Protein kit with bovine serum albumin as a standard. The copper ions in an alkaline medium (biuret reagent) react with peptide, producing a purple color, whose intensity is proportional to the concentration of proteins in the samples being measured in a spectrophotometer at 545 nm.

Statistical analysis

All results are reported as median (range) and presented as box-plot graphics for the different grouP of patients. Hemoglobin, LPI and ferritin levels are presented as mean ± SD deviation. A Shapiro-Wilk test was performed to assess the normality of data distributions, and Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison tests were used when appropriate. Spearman's correlational analyses were also performed between variables. For all analyses, we used a GraphPad Prism 5.0 software, and a $P < 0.05$ was considered significant.

RESULTS

Patient characteristics are presented in Table 1. Electrophoretic analyses indicated that all subjects had normal hemoglobin profiles (data not shown). As expected, LPI and ferritin levels were higher in the transfused subjects. Specifically, subjects receiving five or more transfusions had significantly higher LPI levels ($P < 0.05$), and patients receiving more than ten transfusions had significantly higher ferritin levels ($P < 0.05$) compared to controls. Additionally, we found that the number of transfusions was significantly correlated with LPI and ferritin levels ($P < 0.05$) (Table 2).

The OS markers TBARS (Figure 1A), protein carbonyl (Figure 1B) and DCFH-DA oxidation (Figure 1C) were all significantly higher in transfused subjects compared to the control group ($P < 0.05$). However, total thiol levels were significantly lower in subjects receiving more than ten transfusions compared to controls ($P < 0.05$) (Figure 1D).

The activity CAT and GPx were significantly lower in subjects receiving five or more transfusions compared to controls ($P < 0.05$) (Figure 2A and C). SOD activity was significantly lower in subjects receiving more than ten transfusions compared with controls ($P < 0.05$) (Figure 2B). Furthermore, significant negative correlations were observed between the number of transfusions and the activity of these antioxidant enzymes ($P < 0.05$) (Table 2).

Additional correlations were found between LPI levels and OS markers ($P < 0.05$), with the exception of DCDH-DA oxidation (Table 2). LPI and ferritin were negatively correlated with antioxidant enzyme activities and total thiol, and positively correlated with carbonyl and TBARS levels (all $P < 0.05$). Indeed, it was found that antioxidant enzyme activities were positively correlated with total thiol levels, and negatively correlated with the levels of protein carbonyl and TBARS ($P < 0.05$). In contrast, protein carbonyl and TBARS levels were positively correlated ($P < 0.05$).

DISCUSSION

Our data are in accordance with a previous study showing that the increase in LPI content could lead to an increase in ROS generation, and consequently an increase in oxidative damage[12]. Additionally, based on data concerning hemoglobin profile, we discarded hemoglobin disorders in these individuals. These data are extremely important to avoid misinterpretations, as it was previously shown that any imbalance between α and β chains of hemoglobin (α or β-thalassemia, respectively) plays a crucial role in OS[27]. Besides, there are data linking the observed levels of the various biomarkers evaluated in this study to health outcomes, such as in renal failure[28] and breast cancer[29].

Taking into account our results and those previously found, it is plausible to assume that under blood transfusion therapy, the excess of labile (catalytically active) iron must generate free radicals *via* Fenton chemistry, resulting in oxidative damage to biomolecules *in vivo*[30]. Our assumption is further supported by a previous report showing that iron-catalyzed ROS generation leads to an increase in the genomic instability in hematopoietic progenitor cells[31]. Moreover, it was shown in animal models that iron overload causes liver damage *via* both oxidative and nitrosative mechanisms[32]. Indeed, we assume that under repeated blood transfusions, the iron content increases to values that overwhelm the protective mechanisms, leading to an increase in the amount of iron available to form complexes with small molecules, the

Table 2 Spearman's correlations between biochemical and oxidative markers in polytransfused subjects

	LPI	Ferritin	GPx	SOD	CAT	TBARS	DCHF-DA oxidation	Carbonyl	Total thiol
Transfusion number	0.8569[b]	0.7991[b]	-0.8796[b]	-0.7103[b]	-0.8143[b]	0.5114[b]	0.0111	0.5793[b]	-0.5555[b]
Total thiol	-0.4151[b]	-0.3354[b]	0.4830[b]	0.4849[b]	0.5401[b]	-0.2790[b]	0.0106	-0.1164	-
Carbonyl	0.5583[b]	0.5122[b]	-0.5613[b]	-0.3713[b]	-0.4862[b]	0.5208[b]	-0.0627	-	-
DCHF-DA oxidation	-0.1291	-0.0049	0.0370	-0.0446	-0.0401	-0.2293[a]	-	-	-
TBARS	0.4984[b]	0.4144[b]	-0.4638[b]	-0.3113[b]	-0.3457[b]	-	-	-	-
CAT	-0.7266[b]	-0.5944[b]	0.8945[b]	0.7251[b]	-	-	-	-	-
SOD	-0.6085[b]	-0.5744[b]	0.7443[b]	-	-	-	-	-	-
GPx	-0.7973[b]	-0.7144[b]	-	-	-	-	-	-	-
Ferritin	0.9112[b]	-	-	-	-	-	-	-	-

[a]$P < 0.05$, [b]$P < 0.001$ *vs* DCHF-DA oxidation. CAT: Catalase; DCHF-DA: 2',7'-dichlorodihydrofluorescein diacetate; GPx: Glutathione peroxidase; LPI: Labile plasmatic iron; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid-reactive substances.

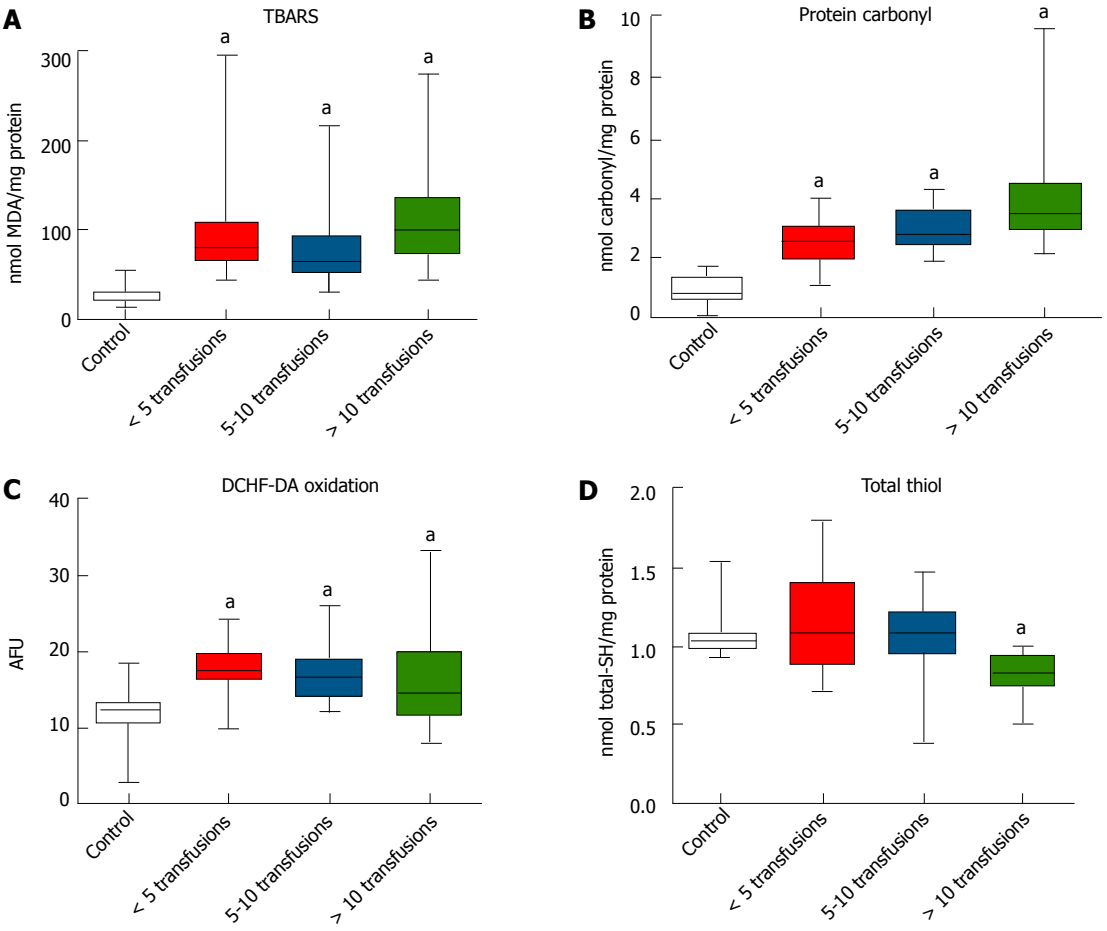

Figure 1 Oxidative stress markers in transfusion patients. A: Thiobarbituric acid-reactive substances (TBARS); B: Protein carbonyl; C: 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) oxidation; and D: Total thiol levels in controls (n = 15), those receiving < 5 transfusions (n = 14), 5-10 transfusions (n = 11), and > 10 transfusions (n = 14); [a]$P < 0.05$ *vs* controls.

"catalytically active iron complexes". Thus, we assume that the ROS generated are responsible for the oxidation of DCHF-DA found in the transfused subjects, which is supported by a previous report showing that overload with iron (ferric nitrilotriacetate) leads to an increase in DCHF-DA oxidation in cultured rat hepatocytes[33].

Interestingly, we found some changes in the OS parameters even in the absence of significant iron accumulation, suggesting that alterations in OS markers could precede iron accumulation in patients following blood therapy. Accordingly, it seems logical that the differences in other parameters, such as hemoglobin and ferritin levels, could potentially contribute to the different oxidative state among patients. Thus, it is difficult to affirm that iron alone is the primary factor responsible for these differences. This point is extremely relevant and deserves further attention in future investigations.

The results of this study also show that levels of

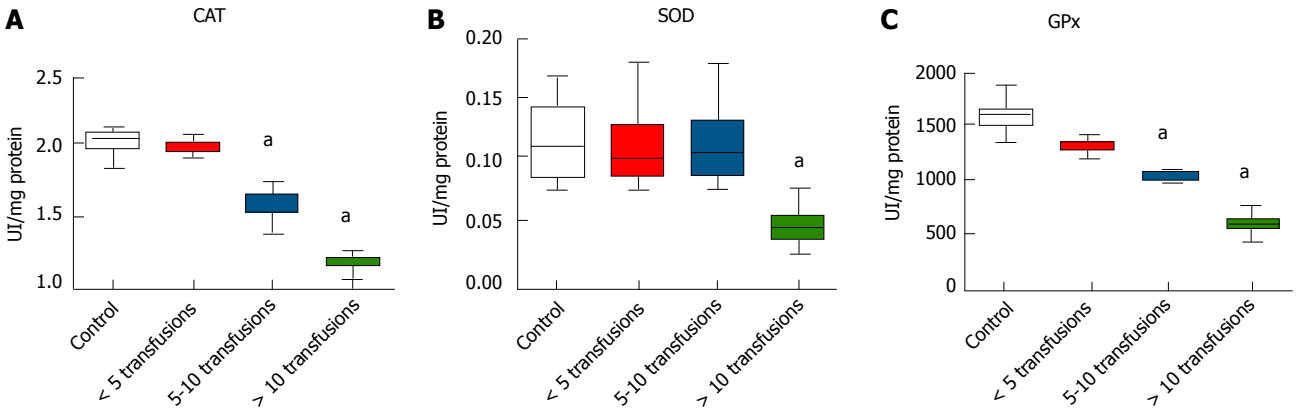

Figure 2 Antioxidant enzymes in transfusion patients. A: Catalase (CAT) activity; B: Superoxide dismutase (SOD) activity; and C: Glutathione peroxidase (GPx) activity in controls (n = 15), those receiving < 5 transfusions (n = 14), 5-10 transfusions (n = 11), and > 10 transfusions (n = 14); $^{a}P < 0.05$ *vs* controls.

TBARS significantly increased in subjects receiving blood transfusions, which was positively correlated to LPI content, ferritin content and the number of transfusions. These findings are in accordance to previous reports showing that the levels of lipid peroxidation products were increased in β-thalassaemic patients receiving blood transfusions[19] and in subjects with hepatic iron overload[6]. Moreover, we found a significant increase in the protein carbonyl in the subjects receiving repeated blood transfusions, which was correlated with LPI content. Additionally, our data are in accordance to a previous paper showing a significant increase in the protein carbonyl content associated with iron overload[33].

A significant reduction in total thiol levels was found in the subjects receiving repeated blood transfusions, which is consistent with a report showing a decrease in thiol content in the liver of rats treated with iron[34]. Albeit not completely understood, we believe that thiols are oxidized (consumed/used) in these subjects due to OS status following iron overload. Another possibility is that the iron could react non-enzymatically with thiols in plasma to generate ROS, which directly leads to reduction of antioxidant capacity in plasma and the increased susceptibility of blood components to oxidation[35]. Thus, this thiol-dependent free radical generation by iron overload might be a potential contributing factor for the changes in the oxidative markers reported here. Our assumptions are supported by a study showing that oxygen radicals can be produced by iron-catalyzed auto-oxidation of cysteine or glutathione[36]. Therefore, the generated ROS (either by Fenton chemistry or by iron-catalyzed auto-oxidation of thiols) may be responsible for the oxidation of other biomolecules reported here, such as lipids and proteins.

The results of the present study demonstrate a marked decrease in antioxidant enzyme activity in the subjects with iron overload, which is consistent with previous reports[30,33]. Moreover, enzyme activities were negatively correlated with LPI, TBARS and protein carbonyl levels. In line with this, we presume that the decrease in the enzymatic activity of antioxidants further contributed to the OS condition. Indeed, Chakraborty *et al*[19] showed that the decrease in antioxidant enzymes strongly contributes to an increase in OS markers (TBARS, protein carbonyl and ROS). Although our data do not support this supposition, we hypothesize that a decrease in the antioxidant enzymes reported here could, at least in part, be due to a decrease in their expression. Indeed, it was previously shown that both CAT and GPx were downregulated under OS conditions in human cells[37]. However, the mechanisms regulating the expression of antioxidant enzymes under iron overload remain to be explored in more detail.

Our data confirms the involvement of OS in patients following therapy with repeated blood transfusions. Additionally, we found that the changes in the OS markers are correlated with iron content, ferritin and the number of transfusions. Thus, iron chelators that efficiently decrease the levels of labile iron are candidates to counteract the iron-induced ROS generation[38]. However, more studies are necessary to better understand the mechanism(s) associated with iron-induced oxidative changes, to minimize the side effects associated to blood transfusion therapy, and to provide some clinical benefits. As antioxidant supplementation is not entirely safe and may cause unfavorable effects to different patients, more discussion on its potential benefits is warranted[39].

In conclusion, our data confirm the involvement of OS and its correlation with LPI and ferritin in unspecified anemic patients following therapy with repeated blood transfusions. However, we found some alterations of OS markers even in the absence of significant iron accumulation, which encourages us to further explore the changes in the OS parameters that occur before iron overload in subjects receiving blood therapy.

COMMENTS

Background

Iron is an essential element that participates in several metabolic activities of cells. However, in excess, iron can be a cause of oxidative stress (OS) in subjects undergoing blood transfusion therapy. Despite this, the relationship between plasmatic iron content, OS markers and the activity of antioxidant enzymes in anemic subjects receiving repeated blood transfusions remains to be better characterized.

Research frontiers

Blood therapy has been used in medical practice to treat anemic patients. However, the increase in the iron level in patients following blood therapy must be considered. Thus, the purpose of this research was to better understand the changes associated with OS markers in patients undergoing blood therapy in order to prevent iron-supported oxidative damage in anemic subjects.

Innovations and breakthroughs

Previous data have shown that blood therapy is associated with iron overload, and consequently, with oxidative changes in various tissues. However, efficient therapies to prevent the side effects associated with repeated blood transfusions are not known. Thus, elucidative studies regarding the plasmatic oxidative changes associated with iron overload are necessary. Here, the authors found that anemic subjects undergoing transfusions show increased levels of plasmatic labile iron, protein carbonyl, thiobarbituric acid reactive substances, and 2',7'-dichlorodihydrofluorescein diacetate oxidation, as well as decreased total thiol levels. Additionally, the activities of superoxide dismutase, catalase, and glutathione peroxidase were significantly lower in the transfused subjects. Significant correlations were found between the number of transfusions, plasmatic iron content, OS markers and the activity of the antioxidant enzymes.

Applications

The results of this study suggest that antioxidants could be associated with blood therapy. Additionally, iron chelators that efficiently decrease the levels of labile iron could be used to counteract the iron-induced generation of reactive oxygen species. However, more studies are necessary to better understand the mechanism(s) associated with iron-induced oxidative changes in order to minimize the side effects associated with blood transfusion therapy and to provide clinical benefits.

Peer review

This is a study that contains important information regarding iron accumulation in anemic subjects receiving repeated blood transfusions and its correlation with the plasmatic oxidative damage markers in these subjects.

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REVIEW

Aging: A mitochondrial DNA perspective, critical analysis and an update

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Abstract

The mitochondrial theory of aging, a mainstream theory of aging which once included accumulation of mitochondrial DNA (mtDNA) damage by reactive oxygen species (ROS) as its cornerstone, has been increasingly losing ground and is undergoing extensive revision due to its inability to explain a growing body of emerging data. Concurrently, the notion of the central role for mtDNA in the aging process is being met with increased skepticism. Our progress in understanding the processes of mtDNA maintenance, repair, damage, and degradation in response to damage has largely refuted the view of mtDNA as being particularly susceptible to ROS-mediated mutagenesis due to its lack of "protective" histones and reduced complement of available DNA repair pathways. Recent research on mitochondrial ROS production has led to the appreciation that mitochondria, even *in vitro*, produce much less ROS than previously thought, automatically leading to a decreased expectation of physiologically achievable levels of mtDNA damage. New evidence suggests that both experimentally induced oxidative stress and radiation therapy result in very low levels of mtDNA mutagenesis. Recent advances provide evidence against the existence of the "vicious" cycle of mtDNA damage and ROS production. Meta-studies reveal no longevity benefit of increased antioxidant defenses. Simultaneously, exciting new observations from both comparative biology and experimental systems indicate that increased ROS production and oxidative damage to cellular macromolecules, including mtDNA, can be associated with extended longevity. A novel paradigm suggests that increased ROS production in aging may be the result of adaptive signaling rather than a detrimental byproduct of normal respiration that drives aging. Here, we review issues pertaining to the role of mtDNA in aging.

Key words: Mitochondrial DNA; Reactive oxygen species; DNA damage; DNA repair; Somatic mtDNA mutations; Antioxidants; Reactive oxygen species signaling; Mitochondrial DNA degradation; Electron transport; Aging

Core tip: The notion of reactive oxygen species (ROS) -mediated accumulation of mutations in mitochondrial DNA (mtDNA) as a driving force behind aging is increasingly losing ground forcing a revision of the Mitochondrial Theory of Aging. While mitochondrial involvement remains in the center of attention of aging research, the focus is shifting from mtDNA mutations to mitochondrial physiology. The positive effect of increased ROS production on longevity is increasingly viewed as evidence that increased ROS production in aging may be adaptive rather than maladaptive. This novel paradigm explains failure of antioxidants to delay aging in clinical trials.

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INTRODUCTION

While there is no universally accepted definition of the aging process, it is often defined as changes (mostly detrimental) that occur in organisms during their lifespan. Most researchers agree that aging is: (1) universal; (2) intrinsic (*i.e.*, "built-in"); (3) progressive; (4) deleterious; and (5) irreversible. The universality of the aging process suggests the existence of an equally universal mechanism or mechanisms that govern it. Over time, different aging theories proposed a variety of such basic mechanisms. Perhaps the most popular of these theories was (and, arguably, remains) the Free Radical/Mitochondrial Theory of Aging (henceforth MTA) first proposed by Harman[1] in 1956. Initially, this theory simply postulated that aging results from the accumulation of oxygen free radical [reactive oxygen species (ROS)] damage to cellular components, including nucleic acids[1]. Over the years, the theory was refined by, first, the identification of mitochondria as both the source and the target of the ROS[2], and, then, the identification of mitochondrial DNA (mtDNA) as a tally-keeper for the damage. The latter concept was introduced by Fleming *et al*[3] and Miquel *et al*[4,5], and is of particular importance because it provided an answer to critics who questioned the capability of other mitochondrial macromolecules such as lipids, proteins, or RNA to accumulate longitudinal damage over an organism's lifetime. Unlike damage to other macromolecules, damage to mtDNA can be converted to point mutations and deletions, which can be transmitted to and accumulated in daughter molecules through the process of replication, enabling deterioration of the integrity of hereditary information over time. It is this damage-sustaining capacity of mtDNA that makes it central to discussions of aging, and it is this property that will be the focus of the current review. Over the years, the MTA underwent many revisions to accommodate new experimental evidence, and thus, there are almost as many versions of it as there are investigators. As Jacobs observed more than a decade ago, "opponents of the hypothesis (MTA) tend to define it in such a narrow and extreme way that it is almost self-evidently falsified by generally accepted facts. Conversely, its proponents are liable to state the theory in such a vague and general way that it is virtually unfalsifiable experimentally"[6]. Here, we review our current knowledge of mtDNA maintenance as it pertains to the MTA, which consists of the following basic tenets: (1) Mitochondria are a significant source of ROS in the cell; (2) Mitochondrial ROS inflict damage on mtDNA; (3) Oxidative mtDNA damage results in mutations; (4) mtDNA mutations lead to the synthesis of defective polypeptide components of the electron transport chain (ETC); (5) Incorporation of these defective subunits into the ETC leads to a further increase in ROS production, initiating a "vicious" cycle of ROS production, mtDNA mutations, and mitochondrial dysfunction (Figure 1). This tenet appears to be the most controversial, and is no longer recognized as a part of the MTA by many researchers[7]; and (6) Eventually, mtDNA mutations, ROS production and cellular damage by ROS reach levels incompatible with life.

Some recent experimental evidence has called into question the validity of the MTA, prompting its reevaluation (see *e.g.*,[7]). Here, we present a historical perspective of our views on the role of mtDNA in aging and update our earlier critical review of the topic[8].

MTDNA

MtDNA (Figure 2) in mammals is a circular molecule that encodes 37 genes, including 2 rRNAs, 22 tRNAs, and 13 polypeptides. All 13 polypeptides are components of the oxidative phosphorylation (OXPHOS) system. They are encoded using a non-standard genetic code, which requires its own translational machinery separate from that of the nucleus. Two rRNAs and 22 tRNAs involved in this mitochondrial protein synthesis are also encoded by mtDNA. Mitochondrial DNA is densely packed into nucleoids, each containing as few as 1-2 mtDNA molecules[9].

A significant body of indirect evidence implicating mtDNA in longevity was contributed by studies on the inheritance patterns of longevity, which suggested possible cytoplasmic (mitochondrial) inheritance[10], and from studies which revealed the association of some mtDNA variations with longevity[11-14]. However, other studies indicate that these associations are weak[15]. The latest large-scale study on mtDNA and aging suggests that the relationship between mtDNA variants and longevity may be much more complex, and that while mutations in the OXPHOS complex I may beneficially affect longevity, the coincidence of mutations in complexes I and Ⅲ as well as the simultaneous presence of mutations in complexes I and V are detrimental. These more complex relationships escape detection by haplogroup analysis and require sequencing of complete mitochondrial genomes[16]. Overall, these findings indirectly support the idea that mtDNA variations may contribute to longevity.

MITOCHONDRIA ARE A SIGNIFICANT SOURCE OF ROS IN CELLS

ROS generation by mitochondria

In the course of their migration through the respiratory chain, electrons can "escape" and participate in the single-electron reduction of oxygen resulting in the formation of the superoxide radical ($O_2^{\bullet -}$ Eq. 1). The detailed overview of this process is presented elsewhere[8,17]. While the exact magnitude of ROS production *in vivo* re-

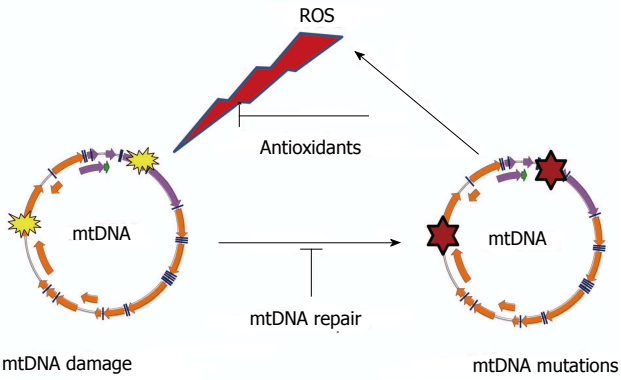

Figure 1 "Vicious cycle" of reactive oxygen species production, mitochondrial DNA damage, mitochondrial DNA mutagenesis and further reactive oxygen species production. The cycle implies an exponential growth of reactive oxygen species (ROS) production and mitochondrial DNA (mtDNA) mutagenesis.

mains debatable, we and others repeatedly argued[8,17] that the values of 1%-2% of total oxygen consumption[18] frequently cited in the literature are not reflective of physiological conditions and that the real rates are much lower.

ROS are produced by multiple sites in mitochondria[19]. Sites other than complexes I and Ⅲ are rarely mentioned in the context of aging. However, recent data suggest that some of these sites may have higher ROS production capacity than respiratory chain complex Ⅰ, which is often viewed as a major source of matrix superoxide production[20]. Moreover, it was argued that the endoplasmic reticulum and peroxisomes have a greater capacity to produce ROS than mitochondria do[21]. Another important consideration is that O_2•- produced by the mitochondrial respiratory chain inactivates aconitase, thus suppressing the Krebs cycle and reducing supply of NADH and $FADH_2$ to the respiratory chain. This can reduce electron flow through ETC, lower the reduction of ETC complexes, and diminish the production of O_2•-[22,23]. Thus, O_2•- production by ETC may be regulated by a negative feedback loop. Finally, actively respiring mitochondria may consume more ROS than they are capable of producing[24].

Mitochondrial ROS neutralization

ETC-generated ROS are detoxified through a two-step process. First, O_2•- is converted to H_2O_2 either spontaneously, or with the help of superoxide dismutases (Eq. 2). Two superoxide dismutases were described in mitochondria: SOD2 in the matrix and SOD1 in the intermembrane space. Interestingly, there is evidence of SOD1 activation by O_2•-[25]. The relative stability and membrane permeability of H_2O_2 ensure its ready access to mtDNA, yet like O_2•- this ROS is unable to efficiently react with DNA[8]. Only when H_2O_2 undergoes Fenton chemistry in the presence of transition metal ions (Eq. 3) is it converted to the extremely reactive hydroxyl radical. This ROS can efficiently damage mtDNA and other mitochondrial components[26,27]. At the second step, H_2O_2 in the mitochondrial matrix is detoxified by peroxiredoxins Ⅲ and Ⅴ (Prx Ⅲ and Prx Ⅴ, Eq. 4 and 5, respectively[28]) and by glutathione peroxidase 1 (GPx1, Eq. 6). Of the eight known GPx isoforms, this one is targeted to the mitochondrial matrix[29]. Another isoform, GPx4, is involved in detoxification of the mitochondrial membrane hydroperoxides[30] and is relevant due to the close association between mtDNA and the inner mitochondrial membrane. Prx Ⅲ is about 30-fold more abundant in mitochondria than GPx 1[31]. It is generally believed that catalase does not localize to mitochondria[32]. Therefore, GPx 1, and Prx Ⅲ and Ⅴ appear to be the main contributors to H_2O_2 detoxification in the mitochondrial matrix.

$O_2 + e^- \rightarrow O_2^{\bullet -}$ (Eq. 1)

$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$ (Eq. 2)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\bullet}OH + OH^-$ (Eq. 3)

$H_2O_2 + 2\text{Prx Ⅲ}\ (SH)_2 \rightarrow 2H_2O + \text{Prx Ⅲ}\ (SH)\text{-S-S}(SH)\text{Prx Ⅲ}$ (Eq. 4)

$H_2O_2 + \text{Prx Ⅴ}\ (SH)_2 \rightarrow 2H_2O + \text{Prx Ⅴ}\ (S\text{-}S)$ (Eq. 5)

$H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$ (Eq. 6)

Remarkably, the thioredoxin/peroxiredoxin system is capable of detoxifying extramitochondrial H_2O_2 in a respiration-dependent manner, providing evidence that mitochondrial OXPHOS is involved not only in the production of ROS, but also in their detoxification, and raising the question of whether mitochondria *in vivo* are a net source or a net sink of ROS[24].

MTDNA DAMAGE BY ROS

The reaction of O_2•- with non-radicals is spin forbidden[33-37]. In biological systems, this means that the main reactions of O_2•- are with itself (dismutation) or with another biological radical, such as nitric oxide. Therefore, direct reactions of O_2•- with mtDNA are unlikely. This ROS is far more likely to undergo dismutation to H_2O_2 (Eq. 2). As indicated above, H_2O_2 in the presence of transition metal ions, in particular Fe^{2+} and Cu^+, can undergo Fenton chemistry to form the extremely reactive •OH. Mitochondria are rich in iron, as many mitochondrial enzymes possess heme groups and iron-sulfur clusters in their active centers, and this abundance of iron may favor •OH production[38]. Therefore, it has been argued that mitochondria may be particularly susceptible to •OH -mediated oxidation, which plays a major role in DNA oxidation[39]. In this respect, it is important to note that mitochondrial iron is not free, but chelated (bound). Some experimental evidence does support the availability of chelated iron for Fenton-type reactions[40,41], and it is also true that iron chelators like desferrioxamine can efficiently suppress DNA mutagenesis by Fenton chemistry *in vitro*[42]. However, there is still a need for studies that could directly assess the ability of the iron bound in mitochondrial heme- and Fe-S proteins to promote generation of •OH.

Is mtDNA more sensitive to damage?

The mitochondrial genome accumulates germline mutations approximately one order of magnitude faster than

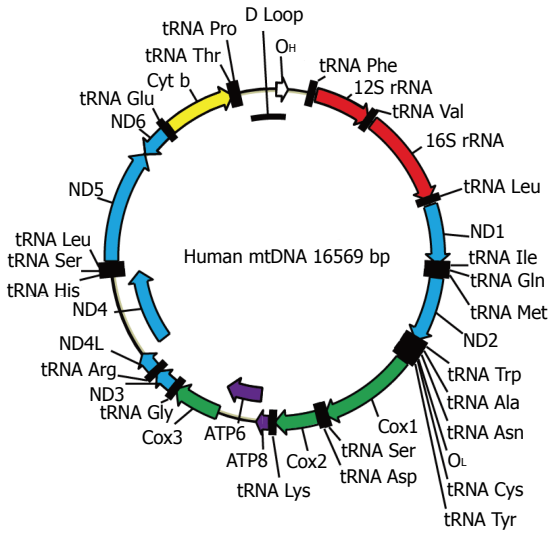

Figure 2 The map of human mitochondrial DNA. OH and OL: Origins of heavy and light strand replication, respectively; ND1-ND6: Subunits of NADH dehydrogenase (ETC complex I) subunits 1 through 6; COX1-COX3: Subunits of cytochrome oxidase subunits 1 through 3 (ETC complex IV); ATP6 and ATP8: Subunits 6 and 8 of mitochondrial ATPase (complex V); Cyt b: Cytochrome b (complex III); ETC: Electron transport chain.

nuclear DNA (nDNA)[43-45]. To evaluate relative accumulation of somatic mutations in nDNA *vs* mtDNA, we used 6×10^{-8} per nucleotide per cell division as an upper estimate for the rate of nDNA mutagenesis (8). Considering that the number of cells in the human body is 3.72×10^{13} (9), which roughly corresponds to 45 cell divisions starting with a fertilized egg, we arrive at $6 \times 45 \times 10^{-8} = 2.7 \times 10^{-6}$ mutations per base pair for the somatic nDNA mutation burden in an aged human, provided that there is no further nDNA mutagenesis after reaching adulthood. The somatic mtDNA mutation burden has been recently estimated to be 1.9×10^{-5} (10), which is less than 1 order of magnitude higher than the 2.7×10^{-6} just calculated for nDNA. mtDNA is turned over with half-lives of 10-30 d in different tissues (11), and therefore the difference in the rates of spontaneous somatic mtDNA mutagenesis between mtDNA and nDNA on per doubling basis may be even smaller than 1 order of magnitude [because in a 70-year-old human mtDNA has replicated on average (assuming a half-life of 30 d) at least $12/2 \times 70 + 45 = 465$ times compared to 45 times for nDNA, not counting repair synthesis]. Therefore, somatic mutations may accumulate at the same per doubling rate in nDNA as they do in mtDNA, while the cumulative burden of mutations in mtDNA may be one order of magnitude higher than that in nDNA in a 70-year-old individual.

In the literature, three properties of the mitochondrial genome are frequently cited as responsible for this faster rate of mtDNA mutagenesis: (1) Its proximity to the source of ROS (ETC); (2) Its lack of "protective" histones; and (3) A limited repertoire of DNA repair pathways available in mitochondria.

It has been argued, however, that proximity to the source of ROS, by itself, is unable to explain the higher mutation rate of mtDNA, and that some available experimental evidence directly contradicts the notion of the protective role of histones[8]. Observations that mtDNA is covered by TFAM[46] and that at least some prominent oxidative DNA lesions are repaired more efficiently in mitochondria than they are in the nucleus[47] also contradict the above arguments.

Moreover, mitochondria evolved a unique way to deal with excessive or irreparable damage: a pathway for degradation or abandonment of damaged molecules (Figure 3)[48,49]. This pathway is enabled by the high redundancy of mtDNA (hundreds to thousands of copies per cell). MtDNA degradation has been reported in response to both oxidative stress[50-52] and to enzymatically-induced abasic sites[53]. It also has been suggested that substrates for the Nucleotide Excision Repair pathway, which has not been detected in mitochondria, are also mitigated through mtDNA turnover[54,55].

If three of the above mentioned rationales in support of mtDNA's higher susceptibility to (oxidative) damage and mutagenesis are not satisfactorily supported by experimental evidence, what then is the basis for the frequently cited higher (compared to nDNA) susceptibility of mtDNA to oxidative stress? Here, one ought to make a distinction between damage to DNA bases-which may lead, upon replication, to point mutations-and damage to the sugar phosphate backbone. The first report comparing the content of the oxidative DNA base lesion, 8-oxodG, in nDNA *vs* mtDNA indicated that mtDNA may accumulate up to 15 times higher levels of this DNA oxidation product[56]. However, it was later established that this dramatic difference was a technical artefact[57]. Independent studies since confirmed that levels of 8-oxodG are similar in nDNA and mtDNA[58-60]. As far as sugar-phosphate backbone damage is concerned, Yakes and Van Houten[61] reported that in mouse embryonic fibroblasts exposed to H_2O_2, mtDNA accumulates more polymerase-blocking lesions than nDNA. These lesions are predominantly single- and double-strand breaks (SSB and DSB) as well as abasic sites with minor contribution from base modifications such as thymine glycol[50]. However, sugar-phosphate backbone damage may induce mtDNA turnover, thus preventing mutagenesis, rather than inducing it[48,50].

CAN MITOCHONDRIAL ROS INDUCE RELEVANT LEVELS OF MTDNA MUTATIONS?

Experimental evidence in support of the mutagenicity of mitochondrially produced ROS remains scarce. There are more studies attempting to assign oxidative stress as a cause of the observed mtDNA mutations than there are studies of mutations induced in mtDNA by experimental exposure of biological systems to oxidative stress. We were unable to detect a statistically significant increase in the level of mtDNA mutations in cells chronically treated with rotenone, which induces ROS production by inhibit-

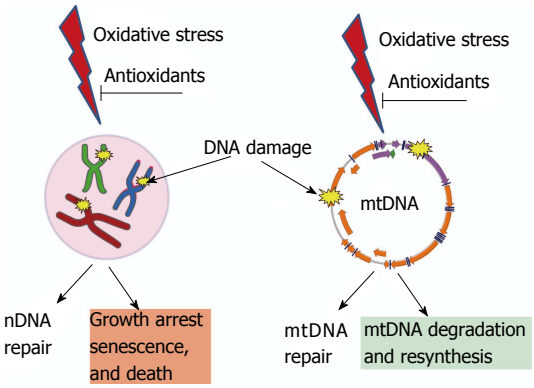

Figure 3 Consequences of unrepaired DNA damage in the nucleus and in the mitochondria. Oxidative damage induces lesions in both nDNA (left) and mtDNA (right). Both nuclei and mitochondria possess DNA repair systems to deal with these lesions. However, cellular consequences of unrepaired damage to nDNA and mtDNA are different. While persistent damage in nDNA results in the activation of cell cycle checkpoints, growth arrest, senescence and death. In contrast, mtDNA molecules with unrepairable damage are simply degraded and new molecules are synthesized using intact molecules as templates. This figure uses Servier elements available under Creative Commons license (155).

ing ETC complex I , and in cells repeatedly exposed to damaging levels of extracellular H_2O_2[50], which suggests that mtDNA is fairly resistant to ROS-induced mutagenesis. Similarly, recent studies indicate that mtDNA mutagenesis is not increased in flies with inactivated SOD and OGG1, an enzyme involved in the repair of oxidatively damaged DNA[62]. In aqueous environments, ionizing radiation induces DNA-damaging ROS: most importantly, the highly reactive •OH. With this in mind, Guo *et al*[63] evaluated 44 DNA blood samples from 18 mothers and 26 children. All mothers underwent radiation therapy for cancer in their childhood, and radiation doses to their ovaries were determined based on medical records and computational models. Sequencing of the entire mitochondrial genome in these patients revealed that the mother's age at sample collection was positively correlated with mtDNA heteroplasmy, a condition in which the cell possesses more than one mtDNA variant (the mitochondrial equivalent of nuclear heterozygosity). However, Guo *et al*[63] failed to detect any significant difference in single nucleotide polymorphisms between mother and offspring. Also, there was no significant correlation between radiation dose to the ovaries and the level of heteroplasmic mtDNA mutations among mothers and children. Therefore, radiation therapy-induced ROS do not appear to contribute, in a substantial way, to mtDNA mutagenesis[63]. This finding is significant because radiation therapy, by design, produces levels of ROS that are much higher than those observed under physiological conditions and therefore have a higher potential to overwhelm cellular antioxidant defenses and produce oxidative damage.

PROPERTIES OF AGING-ASSOCIATED MTDNA MUTATIONS

It is of note that even though age-associated mtDNA mutations are randomly distributed around the genome, there is some bias for the type of mtDNA mutations observed in aging in mitotic *vs* post-mitotic tissues. In mitotic tissues, most common type of mtDNA mutations identified is base substitutions. In contrast, large-scale-deletions are more commonly identified in post-mitotic tissues[64]. Among point mutations in dividing cells, transitions dominate the spectrum (90%) with the remaining fraction of mutations almost equally divided between transversions and small deletions. The frequency of non-synonymous (65.4%) and frameshift/premature termination codons (16.5%) in aging cells is significantly elevated as compared with variants found in the general population (34% and 0.6%, respectively). Also, the predicted pathogenicity of aging-associated mtDNA mutations is higher than that of mutations in the general population[64]. This suggests that human somatic cells, unlike germline cells[65], lack mechanisms to protect them from the accumulation of deleterious mutations.

The advent of Next Generation Sequencing enabled cost-effective interrogation of large numbers of mtDNA bases for mutations. These analyses revealed a minimal contribution of G > T transversions to the spectrum of aging-associated mutations. G > T transversions can be induced by 8-oxodG, a frequently used measure of oxidative DNA damage. This has led some investigators to conclude that oxidative damage does not contribute to aging-associated mtDNA mutagenesis[64,66]. Some observations, however, caution against this interpretation: (1) The most frequent base substitution induced by oxidative stress is a G > A transition[67,68]. This is the most prominent base change observed in mtDNA from aged tissues[66]; (2) 8-oxodG in mammalian cells can also induce G > A transitions[69], and therefore the available evidence does not allow for the complete exclusion of the contribution of this lesion to mtDNA mutagenesis in aging; (3) Cumulative evidence suggests that oxidative stress can induce all possible base substitutions, both *in vitro* and *in vivo*[68], cautioning against basing a conclusion regarding the involvement of oxidative stress in age-related mtDNA mutagenesis solely on an increase in the frequency of G > T transversions. Therefore, in the absence of studies that determine the mutational signature of ROS in mtDNA, any mutation can be interpreted as resulting from oxidative stress. And, conversely, no particular mtDNA mutation can be used, with confidence, as evidence of oxidative stress; (4) It has been shown that oxidative DNA damage does not necessarily lead to an increase in G > T transversions. For example, in DNA oxidatively damaged *in vitro* and passed through bacterial cells, the frequency of G > C transversions was increased, whereas the frequency of G > T transversions was actually decreased as compared to that of untreated DNA[70]. In an almost identical experiment, the frequency of base substitutions at A/T pairs in oxidatively damaged DNA was elevated, whereas the frequency G > T transversions remained unchanged after passing damaged DNA through mammalian cells[42]; and (5) The specific spectrum of oxidative-damage induced DNA mutations is determined, to a great extent, by the particu-

lar properties of the experimental system used (reviewed in[67]). At present, we lack a precise understanding of how oxidative mtDNA lesions are processed by mitochondria to produce mutations. Therefore, no definitive conclusion regarding the contribution of oxidative stress to the spectrum of aging-associated mtDNA mutations can be drawn from the absence of an increase in G > T transversions.

WHAT IS THE FUNCTIONAL SIGNIFICANCE OF AGING-ASSOCIATED MTDNA MUTATIONS?

Given that mtDNA mutations accumulate with aging, are they a cause of (1) mitochondrial dysfunction and/or (2) aging? It is well established that mitochondrial function is only compromised when the fraction of cellular copies of a given mtDNA-encoded gene affected by a given mutation exceeds a certain threshold specific to the mutation (and tissue). This threshold phenomenon can be mediated, at least in part, by intra- and intermitochondrial complementation[71-73]. It is usually accepted that this threshold is 60% to 70% of mutant mtDNA in chronic progressive external ophthalmoplegia and may be close to 95% in the syndromes of mitochondrial encephalopathy, myopathy, lactic acidosis, and stroke-like episodes, and myoclonic epilepsy with ragged red fibers[74]. Therefore, generally, more than 60% of cellular copies for a given mitochondrial gene have to be affected by a pathogenic mutation in order to observe phenotypic manifestation of the mutation[75]. In aging, mtDNA mutations are random, which brings about two caveats. First, not all aging-associated mutations are detrimental. Because of the degeneracy of the genetic code, 25% of mutations will not alter the amino acid sequence of the encoded protein (68.8% of mtDNA encodes for proteins), and others, while causing an amino acid or nucleotide substitution, will not negatively affect the function of the encoded protein or RNA molecule. Second, these mutations are not localized to a particular gene, but rather are randomly distributed among 37 mitochondrially-encoded genes. This means that in order to affect 60% of cellular copies of the largest mtDNA-encoded polypeptide MT-ND5 (which spans 11% of the mitochondrial genome), each mtDNA molecule has to carry on average 0.6/0.11 = 5.45 mutations. For smaller genes, this number will be proportionally higher. Since there is no experimental evidence that supports a selective advantage for deleterious point mutations, both of these caveats suggest that the presence of several aging-associated mutations per mtDNA molecule is required before impairment of mitochondrial function can be observed. These levels are indeed achieved in tissues of mtDNA mutator mice[76,77], but not in naturally aged tissues of experimental animals or humans. Based on the reported frequency of mtDNA mutations, it can be calculated that in mice aged 24-33 mo, mutations affect as little as 20% of mtDNA molecules[78]. Similar calculations using reported values for humans aged 75-99 years[66] suggest that only about 32% of mtDNA molecules are affected by mutations. Therefore, it is highly unlikely that the relatively low mutation loads observed in naturally aged tissues[50,79,80] can account for the observed age-related measurable decline in mitochondrial function and, by extension, cause aging, provided that these mutations are maintained in a heteroplasmic state. Intriguingly, though, some studies indicate that the fraction of respiratory chain-deficient colonocytes in aging mammalian tissues increases after 35 years, and by 70 years of age, up to a third of colonocytes can be respiration-negative. This can be explained by a random genetic drift model. According to this model, multiple rounds of replication may result in the clonal expansion of random mtDNA molecules, leading to a loss of heteroplasmy[81]. In humans, this model predicts that clonal expansion may take decades to occur. Therefore, random drift may provide a satisfactory explanation for the mechanism of respiratory dysfunction observed in aged tissues provided that it can be demonstrated that cell types other than colon epithelium accumulate similar levels of clonally expanded mutations. The random genetic drift in colon epithelium, the tissue in which this phenomenon is best understood, however, appears to be highly heterogeneous, and its extent does not correlate well with chronological age between individuals. For example, a 75-year-old individual may have a lower percentage of respiration-deficient crypts than a 45-year-old[82]. This heterogeneity is inconsistent with the steady and relatively uniform process of aging, and, therefore, argues against random genetic drift being the sole or even a major driving force of aging. It is also unclear whether clonally expanded somatic mtDNA mutations can drive aging in short-lived species. For example, in human colon such mutations are not detectable until about 30 years of age[82]. Can clonally expanded mtDNA mutations explain aging in *Caenorhabditis*. elegans whose lifespan is only 2-3 wk? It is implausible that mtDNA in this organism turns over so much faster to allow for clonal expansion comparable to that observed in humans. Therefore, clonally expanded mtDNA mutations are more likely to be a contributing, rather than driving, factor of aging.

IS THERE EVIDENCE FOR THE EXISTENCE OF THE "VICIOUS" CYCLE?

As noted above, "vicious" cycle is the most contentious part of the MTA. The main premise of the "vicious" cycle hypothesis is the existence of a feed-forward cycle of ROS production and mtDNA mutation. That is: (1) increased ROS production in aging leads to increased mtDNA mutagenesis; and (2) increased mtDNA mutation loads result in increased mitochondrial dysfunction and ROS production. The first part of this premise appears intuitive and plausible. Indeed, no antioxidant defense or DNA repair system works with 100% efficiency, and an increase in ROS will inevitably lead to an increase in mtDNA damage and mutagenesis, however little. The

second part of this premise, however, is more contentious. While observations in patients with mitochondrial disease may partially support the notion of increased ROS production in response to increased mtDNA mutation loads, these observations, paradoxically, also refute this notion. First, while some pathogenic mtDNA mutations result in increased ROS production[83,84], this is not a universal property of mutations in mtDNA. This point is best illustrated by observations made in "mito-mice" (mice that age prematurely due accumulation of random mtDNA mutations): these mice accumulate mtDNA mutation loads exceeding those observed in normal aging by more than one order of magnitude, and still this increase does not result in elevated levels of ROS production[76,77,85]. Thus, the majority of mtDNA point mutations will not affect mitochondrial ROS production regardless of their levels. Second, no accelerated aging or increase in mtDNA mutagenesis rates were reported in patients with mitochondrial diseases which are characterized by increased ROS production. Therefore, while increased ROS production is expected to increase the rate of mtDNA mutagenesis, this increase may not be physiologically relevant or experimentally detectable. This second point is relevant to the discussion above regarding threshold levels of mtDNA mutations.

Moraes *et al*[42] argued that if a "vicious" cycle played an important role in the accumulation of mtDNA deletions in somatic tissues, patients with compromised OXPHOS should accumulate mtDNA deletions at an accelerated rate. Their experiments did not support this prediction, leading Moraes *et al*[42] to the conclusion that a "vicious" cycle is not likely to play an important role in the accumulation of age-related mtDNA deletions[86].

To reconcile MTA with the new evidence, Gustavo Barja has put forward a new version of it that does not include the "vicious" cycle. Barja argues that the damage amplification step provided by the "vicious" cycle is unnecessary for the validity of the MTA[7].

ROS PRODUCTION AND LONGEVITY

It is predicted by the MTA that higher ROS production should lead to increased cellular oxidative stress, which should result in increased damage to cellular macromolecules including mtDNA, and ultimately lead to reduced longevity. Conversely, all other conditions being equal, lower ROS production and oxidative stress are expected to be associated with increased longevity. Since the principal contribution of the mtDNA to the aging process, within the framework of the MTA, is through the effects of mtDNA instability on cellular ROS production, it follows that an examination of the role of ROS in aging would be informative. Indeed, the lack of unequivocal evidence establishing a causative role for ROS in aging makes alterations in mtDNA, which are purportedly induced by ROS and contribute to aging by increasing ROS production, irrelevant.

Evidence from animal models

Early on, comparative biology studies established a positive correlation between body size and longevity. More detailed biochemical studies revealed an inverse correlation between mitochondrial ROS production and mtDNA damage on one hand and longevity on the other, across different biological taxa (reviewed in ref[7]), which is in agreement with the MTA. Unexpectedly, and conflicting with the predictions of the MTA, antioxidant defenses also correlated negatively with longevity[87]. Perhaps not surprisingly, an extension of this analysis to other species revealed that in many species, long lifespans defied explanation by the tenets of the MTA. One of the most striking examples in this category is that of the naked mole-rat. These animals, about the size of mice, live almost 8 times longer than mice[88,89]. Strikingly, these animals have very unremarkable antioxidant defenses: their glutathione peroxidase levels are 70 times lower than in mice, resembling those of knockout animals[88]. In the absence of compensatory upregulation of other antioxidant systems, this, predictably, leads to higher levels of oxidative damage in these animals: at least 10-fold higher levels of urinary isoprostanes (a marker of oxidative stress), eightfold increased levels of 8-oxodG (increased DNA damage) in the liver accompanied by reduced urinary excretion of 8-oxodG (reduced DNA repair), and high cellular (especially, mitochondrial) protein carbonyls were reported in this study[89]. The fact that naked mole-rats live longer than mice despite this increased oxidative burden (especially in mtDNA and mitochondrial proteins) strongly argues against the role of oxidative damage as a key determinant of longevity.

Another line of evidence against the MTA comes from studies on C. elegans. This organism has five genes encoding different isoforms of the SOD, an enzyme catalyzing the first step in the detoxification of superoxide (Eq. 2). Inactivation of the SOD isoforms in this organism either individually or in groups of three (including inactivation of all mitochondrial isoforms), failed to decrease the lifespan[90]. Instead, inactivation of *sod-2* led to increased longevity, which was associated with increased oxidative damage to proteins. Moreover, an *sod-2* mutation further increased lifespan of long-lived clk-1 mutants. Finally, the same group has recently inactivated all five sod genes in C. elegans and demonstrated that while animals completely lacking any SOD activity are more sensitive to multiple stressors, they have normal longevity[91]. Similarly, inactivation of the major mitochondrial antioxidant system by mutating Prx Ⅲ (Eq. 4) decreased overall fitness in this organism, but failed to affect the lifespan[92].

In the fruit fly, somatic mtDNA mutagenesis was not affected by inactivation of SOD either alone, or in combination with OGG1, an enzyme involved in repair of oxidative DNA damage, even though lifespan was affected[62]. These observations suggest a minimal contribution of oxidative stress to age-related somatic mtDNA mutagenesis.

Mclk1$^{+/-}$ mice heterozygous for the key enzyme in the biosynthesis of ubiquinone, an electron transporter

and mitochondrial membrane antioxidant, demonstrate extended longevity. This genetic defect is accompanied by an impairment of the ETC and by increased mitochondrial, but not cytoplasmic, oxidative stress[93]. Inactivation of the homologous gene *clk-1* in C. elegans also resulted in increased longevity. This led the authors to hypothesize that an increase in the generation of mitochondrial ROS might accompany aging not because ROS play a causal role in this process but rather because ROS stimulate protective and restorative processes that help to counteract age-dependent damage[94,95].

Track record of antioxidant-based life-extending strategies

It is predicted by the MTA that reducing intracellular ROS production should reduce damage to macromolecules, including mtDNA, and ultimately increase longevity. As a result, numerous interventional studies have been performed in both vertebrate and invertebrate models. Treatments in these studies typically included either lifelong supplementation with nonenzymatic antioxidants or genetic manipulation of intracellular levels of enzymatic antioxidants. These studies produced inconclusive results: while in some instances it was possible to achieve a modest increase in longevity, many studies revealed the lack of correlation, or even a negative correlation, between antioxidant defenses and lifespan (reviewed in ref[7]). In some instances, these studies produced different results in different species. For example, mitochondrial expression of catalase was reported to have no effect on the longevity of drosophila[96], but resulted in a modest (17%-21%) lifespan extension in mice[97]. In contrast, in C. elegans, a fivefold increase in longevity was reported for animals carrying two mutations (*daf-2 and clk-1*) in nDNA[98]. This suggests that nuclear genes play a pivotal role in determining longevity. To date, no manipulation of mtDNA or the systems involved in its replication, maintenance, or repair has produced comparable extension of the lifespan.

Howes[99] reviewed the results of antioxidant studies which involved more than 550000 human subjects, and concluded that "not only have antioxidants failed to stop disease and aging but also they may cause harm and mortality, which precipitated the stoppage of several large studies". Recent meta-studies support his findings: Bjelakovic *et al*[100] analyzed the results of 78 studies between 1977 and 2012, involving a total of 296707 participants, and concluded that antioxidant supplements neither reduce all-cause mortality nor extend lifespan, while some of them, such as beta carotene, vitamin E, and higher doses of vitamin A, may actually increase mortality[100]. The most direct interpretation of these findings in the context of the MTA as it pertains to mtDNA is that reduced oxidative damage to mtDNA does not extend longevity.

Caloric restriction (30%-40% reduction in caloric food intake without malnutrition) is frequently cited as the most reliable means of extending lifespan across diverse taxa and is frequently employed as a means to investigate the mechanisms of aging. Its effect is widely attributed to reduced ROS production and mtDNA damage[101]. However, in a recent survey of 41 laboratory mouse strains, 40% caloric restriction shortened lifespan in more strains than in which it lengthened it[102]. Similarly, a recent study by the National Institute of Aging revealed no beneficial effect of caloric restriction on longevity in primates[103,104].

CONCLUSION

Recently, there has been an emergence of experimental data challenging many aspects of the MTA as defined in the Introduction. This, in turn, has resulted in both a growing skepticism towards the role of mtDNA mutations in aging, and in the transformation of some of our views on mtDNA, ROS, and aging. Thus, the increased susceptibility of mtDNA to ROS-induced strand breaks (but not to oxidative base damage) is now viewed as a component of the mitochondria-specific mechanism for the maintenance of mtDNA integrity through abandonment and degradation of severely damaged mtDNA molecules, rather than as a mechanism for accelerated mtDNA mutagenesis (Figure 3). Also, we have begun to appreciate that increased ROS production in aging may represent evidence for adaptive signaling aimed at mitigating detrimental changes, rather than constituting an unwanted but unavoidable byproduct of respiration.

Even though its current status is controversial, it is the MTA that stimulated the research that advanced our understanding of aging and clarified the place of mtDNA in this process. While it is no longer plausible that mtDNA is either the sole or the main determinant of aging, epidemiological studies do still suggest a contribution of mtDNA variation to longevity[16]. Also, it is becoming increasingly obvious that maternally transmitted low levels of germline mtDNA mutations can have a significant impact on health and lifespan[105]. The random genetic drift theory[81] has the potential to reconcile the observed mitochondrial dysfunction in aged organs with the low average levels of mtDNA mutations in some tissues. These and other findings demonstrate that despite dramatic advances, our understanding of the role of mtDNA in aging remains incomplete. This incomplete understanding persists in large part due to our limited ability to manipulate mitochondria in a meaningful way. The lack of approaches to introduce defined base lesions into mtDNA impedes our progress in understanding the specifics of mitochondrial processing of oxidative DNA damage. This, in turn, limits our ability to deconvolute and interpret the spectrum of mtDNA mutations observed in aging.

In the near future there is great promise for further advances in our understanding of mtDNA's contribution to aging. The advent of Duplex Sequencing methodology now makes it possible to determine the mutational signature of oxidative stress in mitochondria, which is one

of the most important next steps in mtDNA research. The dire need for reliable markers of oxidative mtDNA damage is becoming increasingly obvious. Despite concerted efforts[106,107], detection of the widely used marker 8-oxodG remains variable between labs, which has resulted in contradictory reports: both a 20-fold increase[108] and no change[109] in 8-oxodG content in the mtDNA of OGG1 knockout animals have been reported. The development of methods for the determination of both the identity of mitochondrial ROS generated in vivo and the rates of their production would greatly aid in evaluating the interactions between mtDNA and ROS. Finally, a better understanding of the incidence, kinetics, and extent of random intracellular drift of mtDNA heteroplasmy in different tissues is needed for an accurate determination of its possible contribution to mitochondrial dysfunction in aging.

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REVIEW

Combinations of vascular endothelial growth factor pathway inhibitors with metronomic chemotherapy: Rational and current status

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Abstract

Chemotherapy given in a metronomic manner can be administered with less adverse effects which are common with conventional schedules such as myelotoxicity and gastrointestinal toxicity and thus may be appropriate for older patients and patients with decreased performance status. Efficacy has been observed in several settings. An opportunity to improve the efficacy of metronomic schedules without significantly increasing toxicity presents with the addition of anti-angiogenic targeted treatments. These combinations rational stems from the understanding of the importance of angiogenesis in the mechanism of action of metronomic chemotherapy which may be augmented by specific targeting of the vascular endothelial growth factor (VEGF) pathway by antibodies or small tyrosine kinase inhibitors. Combinations of metronomic chemotherapy schedules with VEGF pathway targeting drugs will be discussed in this paper.

Key words: Metronomic chemotherapy; Vascular endothelial growth factor; Angiogenesis; Bevacizumab; Vascular endothelial growth factor receptor

Core tip: Metronomic chemotherapy has the potential to reduce the toxicity of chemotherapy administered with conventional schedules. In addition, understanding of the importance of angiogenesis in the mechanism of action of metronomic schedules provides a rational to combine this type of administration with targeted agents against the vascular endothelial growth factor signaling pathway.

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INTRODUCTION

Metronomic chemotherapy is defined as a chemotherapy treatment that is given more often but in lower doses than conventional chemotherapy[1]. The different administration schedule results in differences in pharmacokinetics of the given drugs and in general lower peak concentrations but more protracted trough concentrations. These pharmacokinetic differences may have implications for anti-tumor efficacy but equally importantly for adverse effects and tolerability of chemotherapy drugs.

Vascular endothelial growth factor receptors (VEGFRs) are a family of tyrosine kinase cell surface receptors that includes VEGFR-1 (also called Flt-1), VEGFR-2 (also called KDR), VEGFR-3 and the two neuropilin co-receptors NRP-1 and NRP-2. These are ligated by six secreted glucoprotein ligands VEGF-A to -E and PIGF

(Placenta Growth Factor). Ligation of the receptors triggers activation of down-stream cascades that include the Ras-Raf-MAPK and the PI3K-Akt pathways. VEGFR signaling leads to loosening of the inter-cellular junctions of endothelial cells and contributes to increased motility and eventually results in promotion of angiogenesis and vascular permeability[2]. The VEGFR system has a physiologic role during development that is usurped by tumors. Hypoxia in the tumor micro-environment is a well-known inducer of VEGF. Its expression is up-regulated by transcription factor [hypoxia-induced factor (HIF)], a factor consisting of two sub-units HIF-1α or HIF-2α and HIF-β. The β sub-unit is constitutively expressed and the α sub-units are up-regulated by hypoxia through a mechanism involving hypoxia-promoted protein stabilization. Hypoxia prevents proline hydroxylation of HIF-α factors interfering with their interaction with ubiquitin ligase (Von Hippel Lindau) and thus prevents them from being ubiquitinated and degraded in the proteasome[3]. As a result HIF-α factors concentration is increased and in association with HIF-β may initiate transcription of more than a hundred target genes, such as VEGF[4]. Other tumor associated events, such as tumor suppressor p53 inactivation and proliferation-promoting cascades activation, induce VEGF.

In this paper we will review the current data on combinations of VEGF and VEGFR inhibitors with metronomic chemotherapeutics in cancer. Drugs that affect other components of the angiogenesis network or inhibit angiogenesis indirectly may constitute rational partners for development in combination with metronomic chemotherapy but will not be discussed here.

COMMONLY USED METRONOMIC CHEMOTHERAPIES

Metronomic chemotherapy that comprises the backbone of combinations with anti-angiogenic agents is most often given in an oral form and thus availability of an oral form of various chemotherapeutics has dictated the development of such combinations, given the considerable greater ease and practicability of administering lower closely spaced in time (*e.g.,* daily) doses by mouth instead of intravenously. All these oral drugs have been developed as monotherapies or combinations with classic chemotherapy drugs previously and have a well-characterized safety profile when used in conventional doses and schedules. Data also exist for metronomic schedules, although in general less extensive than for conventional schedules. Some intravenous (IV) chemotherapy drugs given in lower doses and more frequently schedules, usually weekly, instead of three-weekly, can be considered metronomic.

Most commonly used oral chemotherapies include oral forms of cyclophosphamide, methotrexate, vinorelbine, etoposide and topotecan, the oral alkylator temozolamide and the oral fluoropyrimidines capecitabine and tegafur in combination with uracil. Metronomic oral cyclophosphamide is most commonly dosed at 50 mg per day instead of the more conventional dose of 100 mg/m^2 per day for 14 d every 4 wk incorporated, for example, in one of the versions of CMF and 600 mg/m^2 IV every 3 wk in another CMF version[5,6]. Metronomic oral methotrexate has been used often in combination with metronomic cyclophosphamide in metastatic breast cancer at a dose of 2.5 mg twice weekly as compared with the dose of 40 mg/m^2 of the classic CMF (of note this dose is considered "low" compared to "intermediate" and "high" doses in the grams range used in the treatment of sarcomas and acute leukemias and requiring folinic acid rescue)[7,8]. A usual dose of oral daily etoposide is 50 mg and a proposed dose of daily oral topotecan is 0.8 mg/m^2[9]. A commonly used metronomic dose of temozolomide is 75 mg/m^2 continuously daily compared with 150-200 mg/m^2 for 5 consecutive days every 4 wk that is a more conventional dose[10]. Of interest, the metronomic administration is part of a first line standard regimen in glioblastoma multiforme in combination with radiation therapy[10].

Metronomic administration of chemotherapy has essentially a lower incidence of some of the most serious and problematic adverse effects of conventional doses of chemotherapeutics such as myelotoxicity and serious gastrointestinal toxicity. In many cases metronomic chemotherapy produces significantly less alopecia that, although not life-threatening, is impacting in patients' quality of life.

TARGETED AGENTS AGAINST VEGF AND VEGFRS

The first anti-angiogenic agent targeting the VEGF-A ligand that entered the clinical arena was the recombinant humanized monoclonal antibody bevacizumab. Activity has been shown mainly in combination with classic chemotherapy agents in a variety of tumors such as colorectal cancer, glioblastoma multiforme, ovarian cancer and carcinomas of the lung[11-14]. Not surprisingly bevacizumab is the agent targeting the VEGF/ VEGFR axis most extensively studied also in combination with metronomic chemotherapy.

An agent targeting the VEGF ligand, that was more recently developed, is the VEGF trap aflibercept. This molecule consists of the second immunoglobulin domain of VEGFR-1, the third immunoglobulin domain of VEGFR-2 and the Fc (constant region) of IgG1 type human immunoglobulins. The construct functions as a decoy receptor that captures circulating VEGF-A and PlGF, preventing them from binding and activating their receptors[15]. Aflibercept has been approved for the treatment of metastatic colorectal carcinoma in combination with the FOLFIRI regimen[16] but has not been studied clinically in combination with metronomic schedules.

An increasing number of small molecules tyrosine kinase inhibitors (TKIs) blocking VEGFRs have gained approval for diverse indications and have entered the

clinic. They are less specific than monoclonal antibodies and tend to block other tyrosine kinase receptors and even non-receptor kinases with varying degrees of affinity. The TKI regorafenib, for example, that is approved for the third line treatment of metastatic colorectal cancer, inhibits, in addition to VEGFR, PDGF, FGFR, C-kit, Ret, BRAF and TIE-2[17]. TKIs sunitinib and pazopanib, approved for the treatment of renal cell carcinoma inhibit, in addition to VEGFR, PDGFR and C-kit, while sorafenib, another TKI also approved for the treatment of renal cell carcinoma as well as for hepatocellular carcinoma, inhibits, in addition to these three receptors, non-receptor kinase BRAF[18].

Targeted agents have a different toxicity profile from chemotherapy in general and metronomic administration in particular. A side effect peculiar to all VEGF pathway blocking agents is hypertension[19]. Skin reactions, hypothyroidism and blood coagulation complications are also other adverse effects.

RATIONAL FOR THE COMBINATION

Metronomic chemotherapy is believed to act against cancer cells in an indirect way through anti-angiogenic actions as well as through immune-mediated effects. An experimental demonstration of the importance of angiogenesis for tumor propagation in mice was obtained by using low dose metronomic cyclophosphamide. In these classic experiments metronomic cyclophosphamide inhibited angiogenesis *in vivo* and induced apoptosis of endothelial cells in a mouse experimental model[20]. This was followed by apoptosis of the human drug-resistant leukemia and lung cancer cell xenografts. Combined treatment of mice with metronomic cyclophosphamide and an angiogenesis inhibitor, TNP-470, resulted in complete eradication of drug-resistant human xenografts. Metronomic schedule consisted of cyclophosphamide 170 mg/kg every 6 d while the classic dose was 150 mg/kg in days 1, 3 and 5 every 3 wk. The effect of metronomic doses of vinblastine has been studied in human umbilical vein endothelial cells (HUVEC). Doses of 0.25-1 pM of the drug produced significant decrease in the angiogenic phenotype (proliferation, chemotaxis and adhesion to extracellular matrix components) without an increase in apoptosis of HUVEC[21]. These experiments argue for the relevance of angiogenesis in the effects of metronomic chemotherapy schedules and, in addition, offer the first evidence for the value of adding a targeted angiogenesis inhibitor.

An additional mechanism of anti-tumor action of metronomic chemotherapy is promotion of the immune response against cancer cells. In a model of tumor bearing mice, metronomic paclitaxel was demonstrated to enhance immune responses obtained with immunization with a DNA vaccine against chimeric CTGF/E7[22]. Another study of metronomic cyclophosphamide in mice bearing xenografts of glial origin or syngeneic tumors confirmed an anti-tumor activity that was dependent on recruitment of immune cells in the tumor[23]. Mice with a severe combined immunodeficiency background or defects on perforin could not mount an anti-tumor response to metronomic cyclophosphamide. In addition treatment with a more conventional intermittent schedule of cyclophosphamide produced a weaker and transient immune activation, a fact interpreted by the authors to denote a need for a more sustained immune stimulation for effective immune response production[23]. Interestingly, in this study, combined treatment with inhibitors of VEGFR axitinib, cediranib and AG-028262 and metronomic cyclophosphamide interfered with the ability of the latter to recruit immune cells to the tumors and decreased the response to it. Immune mediated activity of metronomic chemotherapy appears to be particularly dependent on doses and frequency of the used drugs, at least in pre-clinical mouse models[24]. Work from the same laboratory has shown that, in contrast to immune interference, anti-VEGFR agents may have synergistic anti-tumor effects with metronomic chemotherapy by promoting tumor retention of active metabolites such as 4-hydroxy-cyclophosphamide and, at least partially counteracting, the deleterious effect on anti-tumor immunogenicity[25]. In discordance with the above pre-clinical data arguing for a role of metronomic schedules in triggering anti-tumor immunity, a small clinical study of patients with diverse types of cancer treated with conventional or metronomic schedules of chemotherapy has reported an increase in the ratio of regulatory T cells to effector T cells in both types of schedules but this increase was more pronounced with metronomic schedules[26]. Regulatory T cells may blunt the anti-tumor response of the immune system against tumor cells. The study did not aim to compare the implications of these immune effects on clinical outcomes and, in any case, this would be impossible given the small number of patients across different tumor types and different drugs used[26]. Overall the involvement of an immune response to the anti-tumor effect of metronomic schedules of chemotherapy is far from clear but the addition of VEGF pathway-targeting agents could theoretically improve this immune effect by normalizing the tumor vasculature network and thus improving immune cell access to the tumor by preventing the formation of the pathologic tumor-associated convoluted vessel network. This is due to the fact that morphologically abnormal glomeruloid microvascular proliferations and bridged mother vessels remain dependent on VEGF-A signaling after their formation while feeder arteries, draining veins and capillaries in the tumor beds become VEGF signaling-independent[27].

PRE-CLINICAL EVALUATION

Consolidating the above rational, additional pre-clinical evaluation of combinations of metronomic chemotherapies with anti-VEGF targeted agents has been undertaken.

A human xenograft model of neuroblastoma in SCID mice was used to investigate the effect of combination

treatment of metronomic vinblastine with DC101, an anti-VEGFR2 antibody[28]. Each drug alone produced only a transient tumor inhibition. In contrast the combination resulted in sustained xenograft growth inhibition with no signs of resistance development up to seven months on treatment. In addition, some of the animals were followed after discontinuation of the combination treatment and showed no evidence of tumor recurrence. Treatment was well-tolerated and mice did not display any of the usual signs of toxicity such as weight loss, anorexia, dehydration or skin ulcerations. In agreement with the clinical effect, the combination showed more pronounced apoptotic cell death and angiogenesis inhibition than control or monotherapy treatment in histopathologic sections examination[28].

The same investigators expanded the above findings to a human orthotopic breast cancer model of cell lines MDA-MB-231 and MDA-MB-435 and multidrug resistant derivative lines expressing multidrug resistance pgp glucoprotein[29]. Continuous low dose of vinblastine, cisplatin or doxorubicin in combination with the same anti-VEGFR2 antibody resulted in improved and sustained anti-tumor effects compared with the chemotherapeutics alone while the antibody by itself had an intermediate effect.

Human colorectal cancer cell line KM12SM growing as xenograft in nude mice was investigated as a target of conventionally-dosed irinotecan or the same drug given in a metronomic schedule with or without bevacizumab[30]. Both treatments including metronomic irinotecan (with or without bevacizumab) resulted in greater anti-angiogenic effects compared with conventional schedule as measured by microvessel density and CD31 immunostaining and were better tolerated as measured by a decreased weight loss. Moreover the addition of bevacizumab appeared to act additively to metronomic irinotecan further delaying tumor growth compared with irinotecan monotherapy[30].

Another gastrointestinal tumor studied in a preclinical human xenograft model in mice is pancreatic cancer[31]. In this case the combination of metronomic schedule gemcitabine with sunitinib had specific efficacy in decreasing metastatic progression while its effect on the primary tumors was less than the effect with conventional maximal tolerated gemcitabine schedule.

Still in the realm of gastrointestinal tumor models, human hepatocellular cancer xenografts of the Hep3B cell line were studied in immunocompromised mice[32]. Treatment with sorafenib, a drug that is used clinically in the treatment of the disease, eventually led to tumor resistance and increase in tumor burden despite continuous treatment. Co-administration of a metronomic dose of UFT (15 mg/kg per day continuously) could delay the development of resistance and prolong the median survival of mice from less than 3 to 4.5 mo without increased toxicity[32]. In another study from the same group, orthotopically implanted hepatocellular carcinoma xenografts of the same cell line Hep3B were more efficiently controlled by the combination of metronomic UFT or cyclophosphamide and the anti-VEGFR2 antibody DC101 than with either agents alone[33].

In mice bearing human prostate cancer and rat gliosarcoma cell line xenografts, the VEGFR inhibitor axitinib in combination with metronomic cyclophosphamide displayed improved anti-tumor activity in comparison with either drug alone and this despite decreased tumor uptake of the active cyclophosphamide metabolite 4-hydroxy-cyclophosphamide[34,35]. Axitinib was found to possess, in addition to anti-angiogenic, direct anti-tumor cell effects inducing tumor cell apoptosis and this may relate to the fact that it is, similarly to other TKIs, an inhibitor of pathways other than VEGF, as mentioned previously[35].

Ovarian cancer was the subject of a study combining oral topotecan with pazopanib[36]. Topotecan has activity and is currently used clinically in the treatment of this type of cancer while anti-angiogenesis is also a proven beneficial modality for ovarian cancer and thus the combination is of particular clinical interest. Mice bearing human ovarian cancer xenografts in their peritoneal cavity were treated with either drug alone or their combination. Metronomic oral topotecan plus pazopanib was more effective than topotecan alone in maximal tolerated dose or pazopanib alone in reducing tumor burden and in prolonging the survival of the tumor-bearing mice[36]. Similar conclusions were reached in another study of the same combination using additional ovarian cell line xenografts[37].

In conclusion, extensive pre-clinical evidence pinpoints to the activity of metronomic chemotherapy/anti-VEGF pathway inhibitors combinations in a variety of tumor types. This together with the variety of chemotherapeutics and VEGF-targeting agents used in these combinations and producing the same synergistic effect argues for a mechanism that is independent of the specific tumor and underlying molecular lesions that it bears and is consistent with an anti-angiogenic and immune mediated mechanism.

CLINICAL STUDIES

Glioblastoma multiforme (GBM) is the most common and most aggressive malignant primary brain tumor for which the median survival with trimodality treatment followed by temozolomide maintenance is 15 mo with less than 10% of patients alive at 5 years[10]. Since several preclinical models showed up-regulation of VEGF in GBM cell lines, targeting angiogenesis has been a subject of several clinical trials that led to the approval of bevacizumab as monotherapy for recurrent GBM[38]. Few of these studies attempt to explore the role of metronomic chemotherapy in combination with angiogenic agents in this context and have attempted to bring these combinations in the forefront of GBM therapeutics.

In the front line treatment, two phase Ⅲ randomized trials showed that incorporation of bevacizumab

to the traditional radio-chemotherapy regimen with temozolomide followed by bevacizumab until progression improves the PFS, but without any benefit in OS[39]. The Radiation Therapy Oncology Group (RTOG) 0825 trial included 637 patients who were randomized to receive standard chemo-radiotherapy with temozolamide plus either placebo or bevacizumab (10 mg/kg every 2 wk)[39]. All patients had a Karnofsky Performance Scale score of at least 60. There was no statistically significant benefit of the addition of bevacizumab for the OS (15.7 mo *vs* 16.1 mo, $P = 0.21$) but a significant benefit for the PFS (10.7 mo *vs* 7.3 mo, $P = 0.007$) was noted. It is important to note that patients from the placebo group were allowed to cross over at progression, which may explain the lack of benefit in OS.

AVAglio was an industry-sponsored study conducted mainly in Europe[14]. It had a similar design that included provision to crossover on progression. It randomized 921 patients and, similarly to RTOG 0825, showed a PFS benefit for the addition of bevacizumab (10.6 mo *vs* 6.2 mo, $P < 0.001$) but failed to show an OS benefit (16.8 mo *vs* 16.7 mo, $P = 0.10$). The two trials showed contrasting results regarding quality of life (Qol) effect of bevacizumab. In the RTOG study addition of bevacizumab worsened patient neurocognitive function, while AVAglio showed improvement of Qol, prolonged time to Karnofsky Performance Scale score worsening, as well as delay in the initiation of corticosteroid treatment. One possible reason for this discrepancy between the two trials is that the AVAglio study did not incorporate a measure of neurocognitive functioning into its evaluation for Qol outcomes.

Based on the fact of failure to demonstrate benefit in terms of OS, several oncologists believe that there are not enough data to support addition of bevacizumab in front line therapy and reserve its use in large, bulky tumors with significant associated edema. Currently, Japan is the only country to approve the addition of bevacizumab in newly diagnosed GBM (http://www.roche.com/media/ media_releases/med-cor-2013-06-17.htm).

In recurrent GBM, phase Ⅱ studies have demonstrated response rates of 63% and 6 mo PFS of 38% to 46% when bevacizumab is combined with metronomic irinotecan adjusted according to whether patients were taking metabolic enzyme-inducing anticonvulsants[40,41]. In another phase Ⅱ study response rate was 47.3%, including 2 patients with complete response[42]. A phase Ⅱ trial evaluating biweekly bevacizumab with metronomic etoposide in recurrent malignant glioma[43] included 27 (of 59 total) patients with GBM. The six months PFS was 44.4%, but the toxicity was increased with the combination compared with previous reports of bevacizumab monotherapy. Interestingly low expression of VEGF in tumors assessed by immunohistochemistry was correlated with poorer PFS[43]. Another phase Ⅱ trial closed at the interim analysis as the addition of bevacizumab on metronomic chemotherapy (temozolomide or etoposide) was found to be ineffective[44].

There are limited data concerning VEGFR tyrosine kinase inhibitors in GBM. A phase Ⅱ trial has showed that the combination of sorafenib with temozolamide in recurrent GBM in 43 naïve patients for anti-angiogenic treatment is feasible and safe[45]. Twenty-three out of 43 patients had radiologically stable disease or a partial response and the median PFS was 3.2 mo and the median OS was 7.4 mo. Of note, 11% of patients suffered from a grade 3-4 hand foot syndrome.

In ovarian cancer, the activity of anti-angiogenic therapy with bevacizumab is well documented both as monotherapy (RR 21% in a phase Ⅱ study[46]) and as combination with chemotherapy. Several trials[12,47] have shown a clear benefit in PFS after incorporation of anti-angiogenesis therapy in the management of newly diagnosed advanced ovarian cancer treated with carboplatin and paclitaxel, making it a standard of care option in 1st line.

The concept of metronomic treatment with antiangiogenic therapy has been well studied in case of platinum resistant recurrent patients. A randomized phase III study comparing chemotherapy (weekly paclitaxel, weekly topotecan or liposomal doxorubicin) to chemotherapy plus bevacizumab confirmed the benefit of the combination with doubling of median PFS (6.7 mo *vs* 3.4 mo)[48]. Mature OS data presented in ESMO 2013 congress did not show statistically significant OS benefit of the combination ($P = 0.174$), despite a 3 mo survival advantage of the bevacizumab arm (13.3 mo *vs* 16.6 mo)[49]. Interestingly, in the subgroup analysis a more pronounced impact was noticed when the bevacizumab was combined with weekly paclitaxel (13.2 mo *vs* 22.4 mo), a chemotherapy regimen with anti-angiogenic activity, supporting the idea of the efficacy of the concept[49].

Moreover, a recent retrospective trial of bevacizumab and oral metronomic cyclophosphamide in heavily pretreated platinum resistant ovarian cancer, with 66 patients, has shown that the combination is active with a response rate of 42.4%[50].

The activity of weekly topotecan and biweekly bevacizumab in ovarian cancer has been shown in a trial of bevacizumab 10 mg/kg administered on days 1 and 15 and topotecan 4 mg/m^2 on days 1, 8, and 15 of a 28-d cycle until progressive disease (PD) or excessive toxicity[51]. Median PFS and OS were 7.8 and 16.6 mo respectively, with 22 of 40 (55%) of the patients being progression-free for ⩾ 6 mo. Ten (25%) patients had a partial response (PR), 14 (35%) had stable disease (SD), and 16 (40%) had PD.

A phase Ⅰ/Ⅱ study of sorafenib associated with weekly topotecan in patients with platinum-resistant ovarian cancer or primary peritoneal carcinomatosis has shown some efficacy[52]. In this study, 16 patients were enrolled in a phase Ⅰ part and 14 patients in a phase Ⅱ part. The phase Ⅱ regimen consisted of sorafenib 400 mg daily and topotecan 3.5 mg/m^2 weekly on days 1, 8, 15 of a 28 d cycle. There were 5 PR (16.7%), and 14 patients (46.7%) with SD. Nevertheless, the combination of sorafenib and topotecan caused significant toxicity.

Other studies are ongoing evaluating combinations of therapies targeting the VEGF pathway and metronomic chemotherapy in ovarian cancer. For example, a German phase Ⅰ/Ⅱ trial combines metronomic cyclophosphamide with pazopanib[53] and a phase Ⅱ study to open in United Kingdom (NCT01610869) will combine nintedanib (formerly BIBF 1120, a receptor tyrosine kinase inhibitor blocking signaling through VEGFR, PDGFR, and FGFR, also investigated in idiopathic pulmonary fibrosis[54]) and metronomic daily cyclophosphamide in patients with multiply-relapsed advanced ovarian cancer.

In breast cancer, a phase Ⅱ study of 46 patients (19 of whom had previous chemotherapy for metastatic disease) investigated the combination of metronomic cyclophosphamide at a dose of 50 mg daily with capecitabine 500 mg three times daily and bevacizumab 10 mg/kg every two weeks[55]. It showed a response rate of 48% and a clinical benefit rate of 68%. The clinical benefit was more pronounced in hormone receptors positive disease. The treatment was very well tolerated with the only grade 3 or higher adverse effect occurring in more than 10% of patients being hypertension[55]. The same investigators added erlotinib to the above combination in patients with metastatic breast cancer poorly expressing hormone receptors and negative for Her-2 and observed a response rate of 62%[56].

A phase I study with 20 metastatic breast cancer patients and up to four previous lines of treatment associated metronomic cyclophosphamide 50 mg daily with methotrexate 2.5 mg 2 d per week and vandetanib found to have a maximal tolerated dose of 200 mg daily[57]. Toxicity was acceptable and the clinical benefit rate was 25%. A similar combination with cyclophosphamide and methotrexate as the metronomic chemotherapy backbone and bevacizumab instead of vandetanib produced a clinical benefit rate of 31.8% in another study[58].

Several studies have been published showing clinical activity of metronomic schedules in advanced stage non-small cell lung cancer with acceptable toxicity profile. Data on combinations with anti-angiogenic treatments are less abundant and few studies have been published and few are ongoing. A phase Ⅱ study published only in abstract form so far presented data on the combination of two chemotherapeutics at metronomic doses (paclitaxel 80 mg/m^2 weekly three weeks out of four and gemcitabine 200-300 mg/m^2 also weekly three weeks out of four) with bevacizumab (10 mg/kg biweekly). Maintenance bevacizumab was an option for patients with a good tolerance and no progression. The trial showed a median OS of 30 mo and a 2-year OS of 55% in advanced non-squamous lung cancer[59].

A small pilot phase Ⅱ study combined a metronomic oral chemotherapy part of etoposide at 50 mg per day for 14 d of a 21 d cycle and bevacizumab at 5 mg/kg with a more intense part of cisplatin at a dose of 30 mg/m^2 for 3 consecutive days. This was followed by maintenance of erlotinib and bevacizumab in case of stable disease or response[60]. The combination demonstrated a 69% partial response rate and 86% disease control rate (partial response or stable disease). The PFS was 9.53 mo. Toxicity was also significant as expected from a regimen with a more intense component and included grade 3 or 4 myelotoxicity in 15% of patients and grade 3 or 4 GI toxicity in 18%[60].

A phase I study of the combination of metronomic vinorelbine at a starting dose of 40 mg three times per week with sorafenib (NCT00870532) has been completed but results have not been published yet.

Clinical data from studies in other tumor types, although less abundant, also confirm the concept of the combination. A study of patients with hepatocellular carcinoma and Child-Pugh class A liver function combined sorafenib with metronomic tegafur-uracil and showed a clinical benefit rate of 57%[61]. In a study of metastatic colorectal cancer patients who had at least two previous lines of therapy, the combination of cyclophosphamide 50 mg daily with imatinib 400 mg daily and bevacizumab 5 mg/kg every 2 wk was well tolerated and led to prolonged (more than 6 mo) stabilization of the disease in 20% of patients[62]. In a small study that included 15 patients with malignant neuroendocrine tumors mainly of gastrointestinal origin, the combination of temozolomide 100 mg daily with a long acting somatostatin analogue and bevacizumab produced a 64% response rate and 86% clinical benefit rate[63].

THE WAY AHEAD: PREDICTION OF RESPONSE

As witnessed by other cancer treatments such as anti-Her2 antibodies, the presence of a robust biomarker of response greatly facilitates the development and establishment of a drug in the clinic. The absence of such confirmed biomarkers for anti-VEGF treatments and for metronomic chemotherapy hampers their development in the clinic and has certainly contributed to negative results of trials in some type of cancers and moderating their success in others. Extensive investigations on discovering such biomarkers have not succeeded in bringing any biomarker forward to clinical applicability so far. Initial exploratory investigation on the predictive value of VEGF single nucleotide polymorphisms identified two minor alleles associated with bevacizumab response in a breast cancer population but this was not consistently seen in another study also in breast cancer[64]. Circulating stem or endothelial progenitor cells and MRI imaging parameters have been proposed as markers of response to sorafenib and bevacizumab[65,66]. The previously discussed report on metronomic cyclophosphamide, capecitabine and bevacizumab in breast cancer found a higher response of this treatment in patients with higher baseline circulating endothelial cells[55]. K-ras mutations, which predict for lack of response to EGFR targeting therapies in metastatic colorectal cancer, are not predictive of response to bevacizumab despite k-ras being also part of one of the intracellular signal cascades triggered by VEGFR[67]. This

argues for the importance of indirect mechanisms in the cytotoxicity of VEGF treatments. Prediction of which patient will respond to a given metronomic chemotherapy treatment is similarly currently unfeasible.

Another interesting biomarker that has been proposed as a predictor of response to bevacizumab is thrombocytosis[68]. This is a well validated laboratory value already routinely used in the clinic and has been associated with adverse outcomes in a variety of tumor types[69-72]. Interleukin-6 (IL-6), a thrombopoiesis-promoting cytokine, production by the tumor is implicated in the induction of thrombocytosis[70]. IL-6 is concomitantly an angiogenesis-promoting cytokine and thus its presence may denote a particular angiogenic propensity of tumors and dependence, as a result, to angiogenic pathways. In addition, platelets contribute to this effect by carrying additional pro-angiogenic substances in their granules[73]. As a result their number in a particular patient may represent a marker of the tumor dependency to a combination of treatments such as anti-VEGF and metronomic chemotherapy that rely on inhibition of angiogenesis as a mechanism of their action. Platelet proteome has been reported to change after treatment with metronomic cyclophosphamide, methotrexate and vandetanib[58] but it is unknown if the baseline status of this proteome predict for response to treatment. Platelet number and content represent interesting predictive markers for further investigation for the combination. Confirmation of their value and discovery of other useful predictive biomarkers will certainly facilitate a wider adoption of combinations of VEGF therapies and metronomic chemotherapy that could be a valuable option especially in later line of therapy where lower toxicity therapies are needed to fit with the profile of patients with lower performance statuses.

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