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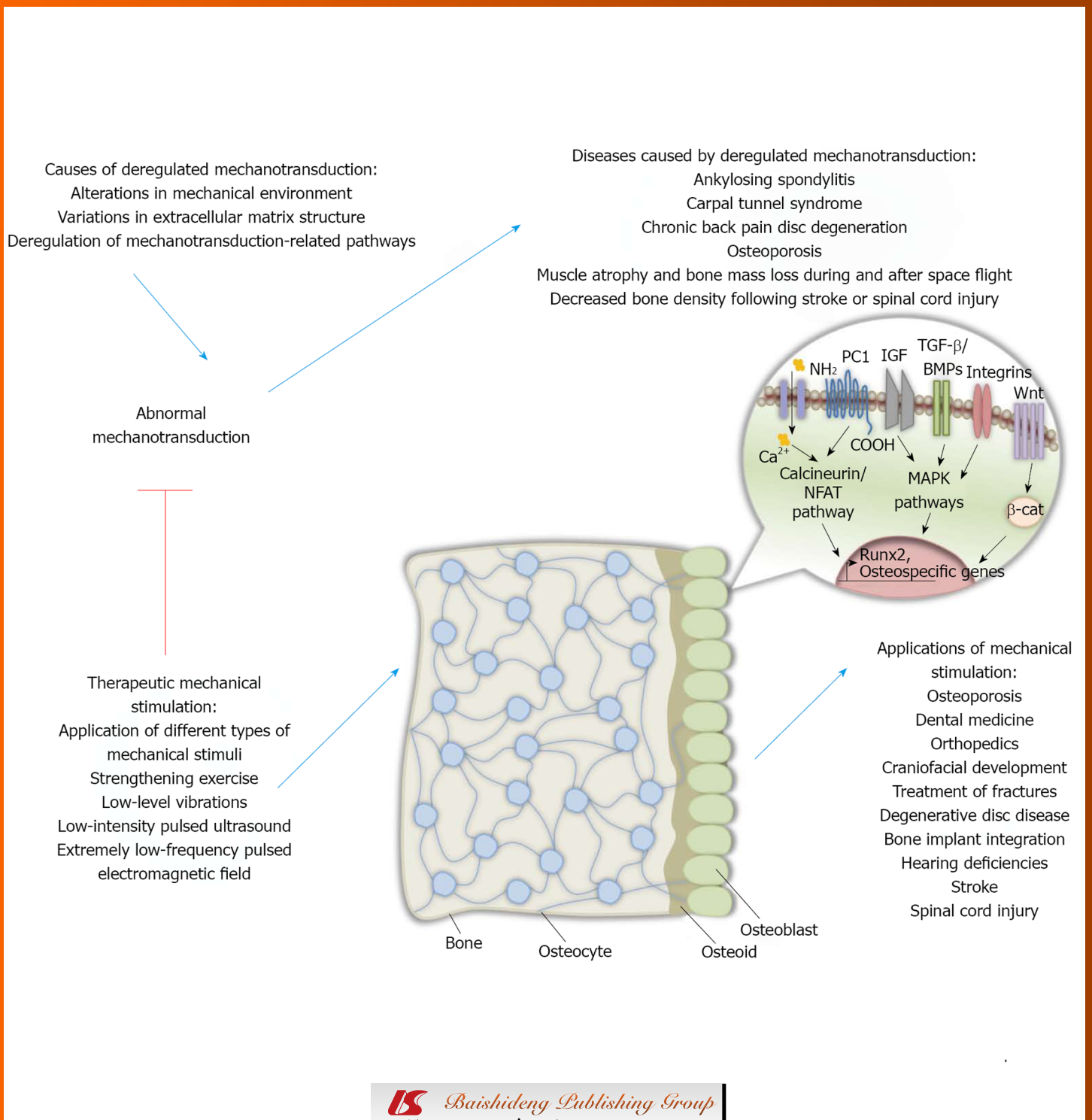

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World Journal of Experimental Medicine
Room 903, Building D, Ocean International Center,
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Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjem@wjgnet.com
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EDITORIAL

Why not change classical treatments for glioblastoma in elderly patients?

Eduardo Perez-Campos, Javier Arjona Perez, Laura Perez-Campos Mayoral, Itandehui Gallegos Velasco, Pedro Hernandez Cruz, Primitivo Gonzalez Olivera

Eduardo Perez-Campos, Department of Biochemistry and Immunology, Technological Institute of Oaxaca, Oaxaca, CP 68030, Mexico
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Itandehui Gallegos Velasco, Pedro Hernandez Cruz, Laboratory of Cancer Glycobiology, Multidisciplinary Research Center, UNAM-UABJO-Medicine, Mexico City, CP 68050, Mexico
Primitivo Gonzalez Olivera, Surgical Oncology, Puebla Mexico, CP 72000, Mexico
Author contributions: Perez-Campos E and Perez-Campos Mayoral L supervised and wrote equally to this work; Arjona-Pérez J, Gallegos Velasco I, Hernandez Cruz P and Gonzalez Olivera P discussed and rewrote the manuscript.
Correspondence to: Eduardo Perez-Campos, PhD, Researcher, Department of Biochemistry and Immunology, Technological Institute of Oaxaca, Zaragoza 213, Oaxaca, CP 68030, Mexico. perezcampos@prodigy.net.mx
Telephone: +52-951-5161140 Fax: +52-951-5140005
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Abstract

In consideration of the poor results obtained with conventional treatments, a review of alternative treatments for elderly patients with glioblastoma was researched in this study. The proposal considers the elimination of human cytomegalovirus, modifying the immune response, arresting growths, blocking some signaling pathways, and modulating the effects of oxygen reactive species.

Key words: Glioblastoma; Treatment; Elderly patients; Glioma; Glioblastoma

Core tip: It is necessary to reconsider the treatment of elderly patients with glioma, focusing on their life quality when conventional treatment is used, such as, chemotherapy and radiotheraphy, in review of the fact that these treatments cause the patient further suffering. This is a review of othe therapeutic options, including some phase I vaccine trials.

Perez-Campos E, Arjona Perez J, Perez-Campos Mayoral L, Gallegos Velasco I, Hernandez Cruz P, Gonzalez Olivera P. Why not change classical treatments for glioblastoma in elderly patients? *World J Exp Med* 2013; 3(4): 50-55 Available from: URL: http://www.wjgnet.com/2220-315X/full/v3/i4/50.htm DOI: http://dx.doi.org/10.5493/wjem.v3.i4.50

INTRODUCTION

Glioblastoma (GBM) is the most aggressive and frequent of all brain tumors. Glioblastoma usually appears between the ages of 45 and 70 years[1]. The goal of surgery is to confirm the diagnosis and reduce the effect of the tumor mass[2]. Survival of elderly patients of more than 71 years, with glioblastoma GBM, is poor. Temozolomide (TMZ) and radiotherapy (RT) improves the average patient survival rate by as much as 10 to 13 mo, in patients of more than 71 years[3].

Stereotactic radiosurgery, whole brain radiation therapy, and surgery, in isolation or in combination[4] with gamma knife, cyberknife, LINAC, stereotactic brachytherapy, boron neutron capture therapy and hadrontherapy, amongst other treatments, have been used as ther-

apy[5,6]. Although all these methods have been reported to improve prognostic indices, when under constant observation, the statistical analyzes of survival are not effective. Radiation therapy has considerable limitations, mainly infiltrating glioma characteristics and neuronal damage.

Although the survival rate is greater when using chemotherapy than when not used[7], the difference in the number of months survived is very low, and the quality of life is much lower when radiation is used rather than with no chemotherapy.

Gliomas show tumor-associated antigens, which should be detectable by the immune system, however, there are shortcomings in the elimination of a tumor.

Gliomas lack clear-cut tumor-rejection antigens for immune targeting by $CD8^+$ T-cells and indicate cancer/testis antigens as NY-ESO-1[8,9]. However, other antigens from human cytomegalovirus (HCMV) have been found.

The question is, how can tumor growth be limited without chemotherapy or radiotherapy? Here, some therapeutic options are reviewed, including some phase I vaccine trials, supporting treatment of elderly patients with glioma (Figure 1).

TREATMENT WITH ANTIVIRAL DRUGS

It has been suggested that glioma in elderly patients should be treated as a tumor associated with viral infection[10], this would mean treatment for the elimination of the infectious agent, arresting growths and apoptosis, modifying the immune response and blocking some signaling pathways, which involve metastasis and the modulation of reactive oxygen species (ROS).

Glioma tissues indicate the change in a cascade of a viral protein, typical of replicative HCMV[11], the virus is trophic in glial cells and the HCMV infection remains in between 50% to 90% of adults. The HCMV can be reactivated when there is inflammation and immunosuppression and plays an active role in the pathogenesis of a glioma[12].

In order to clarify the controversy surrounding glioma and HCMV, some researchers have shown the close relationship between HCMV and glioma in the context of mutations related to their existence[13].

In order to reduce the effects of HCMV on the glioma it is possible to use valganciclovir, which targets the DNA polymerase, or the Cox-2 inhibitor celecoxib, and averts HCMV replication by decreasing PGE2 levels[14]. Moreover, infiltrating gliomas in microglia, have been found to be an important source of PGE2, Cox-2 inhibitors and are an alternative, as opposed to glucocorticoids, in peritumoral edema of malignant gliomas[15]. In addition to its anti-inflammatory properties, celecoxib is able to exert a pro-apoptotic effect *in vitro* and *in vivo* in the absence of the action of Cox-2 in malignant glioma cells. In fact, it has developed a variant of this substance, 2,5-dimethyl-celecoxib, which is more potently cytotoxic against these cells[16]. The effect of celecoxib is dependent on the existence of p53[17].

One mechanism that could be used in the treatment of gliomas, is the induction of autophagy. Valproic acid is a potent histone deacetylase inhibitor which induces cell differentiation, growth arrest and apoptosis in gliomas and other cancers. Valproic acid induces autophagy in glioma cells, independently from apoptosis[18].

Chloroquine and quinacrine bind tightly to nucleic acids, in particular CG sequences, and reinforce its structural configuration and preventing mutagenesis[19]. Chloroquine also acts as an immunomodulator through the inhibition of phospholipase A2 and the tumor necrosis factor-α (TNF-α)[20]. Chloroquine improves survival in patients with GBM when added to conventional therapy[21,22].

TREATMENT BY IMMUNOMODULATION

Gliomas show a sequence of events that increase immunosuppressant cytokines, such as interleukin-10 (IL-10), transforming the growth factor-β (TGF-β), prostaglandin E2, inducing regulatory T cells (Treg), and decreasing costimulatory molecules, all results lose the function of effector T cells. Moreover, GBM cells show human leukocyte antigen (HLA) class I molecule mutation. Loss of HLA class I correlate with the grade of tumor and show little response to immunotherapy. NK cells do not have a histocompatibility complex (MHC) restriction. In patients with GBM, the NK cells are depressed, and it has been observed that when NK cells increase there is tumor regression in a recurrent glioma[23]. Glioblastoma stem cells suppress T cell responses in different ways: producing immunosuppressive cytokine that suppress T cell responses and inducing regulatory T cells, which act as a brake on the immune response and eliminate T cells through apoptosis. This is accomplished through the immunosuppressive protein B7-H1 from stem cells, or soluble galectin-3[24]. Gamma-delta T cells (γδ T-cells) are the primary effector cells in the immune response of a high grade glioma[25].

Some GBM subjects have responded similarly in autoimmune diseases, showing anergy to common bacterial antigens, lymphopenia, defective production of antibodies, and abnormal delayed hypersensitivity [26].

In order to modify the immune response in gliomas, the quantity of Tregs, a subclass of lymphocytes with immunosuppressive properties, is increased. It has been noted that indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurenine, increases the activity of Treg, and could be modified by aciclovir[27]. Also, in order to reduce Tregs and improved antitumoral immunity in other tumors, denileukin diftitox is used, which is a recombinant fusion protein of IL-2 and the diphtheria toxin targeting IL-2 receptors (CD25)[28].

Dendritic cells (DCs) have an antigen presentation function, their maturation is critical for the induction of the T cell response. Glioma cells suppress the matu-

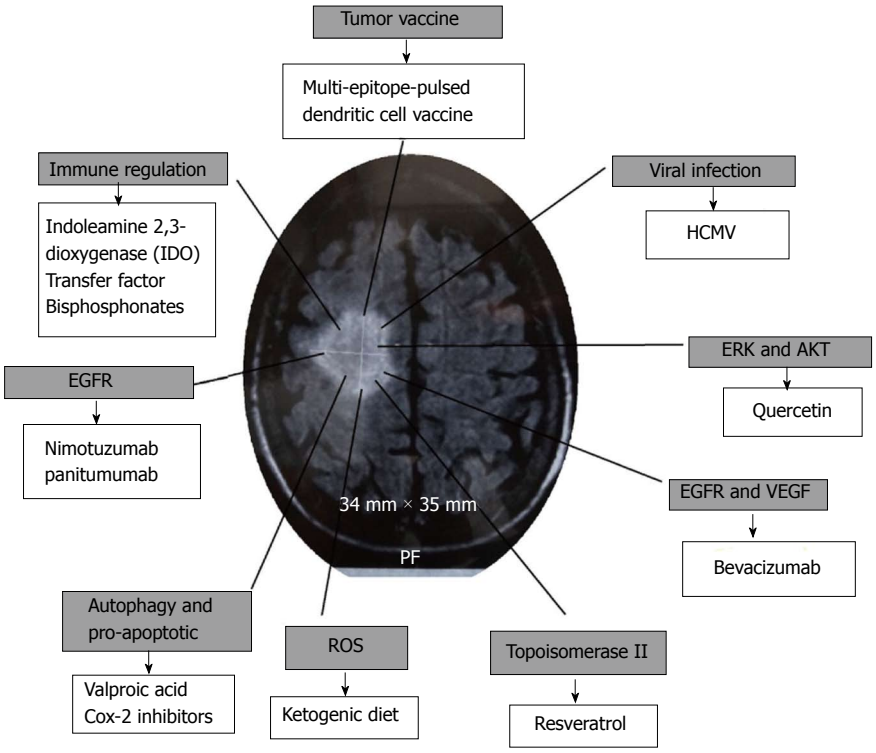

Figure 1 Molecular targets propose for glioblastoma. EGFR: Epidermal growth factor receptor; ERK: Extracellularregulatedproteinkinases; ROS: Reactive oxygen species; VEGF: Vascularendothelialgrowthfactor.

ration of DCs[29], Inmunoferon (AM3) promotes the maturation of DCs derived from human monocytes[30], and reduces the concentration of TNF-α and IL-6[31], IL-6 promoting the invasiveness of glioma cells *via* up-regulation of the STAT3 pathway and fascin-1[32].

Transfer factors are dialyzable products of low molecular weight extracted from the cells involved in the immune system. It has been reported that the transfer factor, in combination with carmustine in experimental malignant glioma, reduces the tumor and increases the $CD2^+$ $CD4^+$, $CD8^+$, NK lymphocytes, and apoptotic tumor cells[33]. γδ T-cells recognize unprocessed non-peptide compounds known as phospho-antigens and are involved through the mevalonate pathway or 1-deoxy-D-xylulose-5-phosphate, in activating the cytotoxic response and releasing cytokine and chemokines[34,35]. γδ T-cells activation can be induced *in vivo* by molecules such as zoledronic acid, which induce the accumulation of the T cell Vγ2. The zoledronic acid induces an effective antitumor response. Aminobisphosphonates play dual roles, apparently acting directly against GBM cells and enhancing antitumor activity from Vγ2 T-cells, which is present up to 75% in γδ T-cells[36]. Otherwise bisphosphonates, such as alendronate, increase γδ T-cell activation by interaction with monocytes circulating or macrophage associated tissues[37].

INHIBITION OF SIGNALING WITH ANTIBODIES AND KINASE INHIBITORS

In glioblastomas there are many genomic alterations especially RTK amplification/mutation, NF1 mutation/loss, NFK1B loss, PIK3R1/PIK3CA mutation, PTEN mutation/loss, TP53 loss, CDK2N2A loss, CDKN2B loss, RB1 mutation/loss, and CDK4 amplification[38].

Heterogeneity in glioblastoma suggests that no therapy can be generalized in different types of GBM. In neural/classical type GBM there are mutations in the epidermal growth factor receptor (*EGFR*) gene. In proneural type GBM frequent mutations occur in p53, in platelet-derived growth factor receptor A, and in isocitrate dehydrogenase 1. Mesenchymal type GBM causes frequent mutation, observed in neurofibromatosis type 1 gene (NF-1)[39].

In neural/classical type GBM, Nimotuzumab could be used, it is a humanized antibody that recognizes EGFR[40] or panitumumab, originally approved for treating colorectal cancer, and it has been used with good results in glioma[41]. In proneural types which have IDH1 mutation, bevacizumab[42] could be used. Bevacizumab is a humanized monoclonal IgG1 antibody that selectively binds with great affinity to human vascular endothelial growth factor. This antibody is being used in phase Ⅲ randomized trials in combination with temozolomide and radiotherapy, and has also been reported to be of benefit in phase Ⅱ studies in recurrent glioblastoma [43]. In mesenchymal type NF-1, therapeutic targets use Ras antagonists and ERK antagonists. Also, mTOR dysregulation and PI3K/PKB/mTOR are central regulators of cell proliferation, growth, differentiation, and survival[44], they could logically be used with resveratrol or quercetin[45]. Treatment with low doses of resveratrol inhibits mono-ubiquitination of histone H2B at K120 in senescent glioma cells[46]. Resveratrol reduces TNF-α induced

NF-κB, and reduces the effect of urokinase plasminogen activator[47]. Resveratrol acts over topoisomerase II on one of the enzymes found in highly proliferating cells[48]. Quercetin causes a rapid reduction in phosphorylation regulated to kinase (ERK) and Akt signaling. With quercetin the death of human glioma cells is brought about with a mechanism that involves caspase-dependent down-regulation of ERK, Akt, and survivin[49].

ROS are regulators of mitogen-activated protein kinase (MAPK), a family of serine/threonine kinases. An increase in intracellular ROS participates in autophagic execution[50]. The ketogenic diet reduces oxygen reactive species (ROS) in tumor cells, it also induces a total pattern of reversal in gene expression, compared with non-tumorous tissues[51].

TREATMENT WITH TUMOR VACCINES

Considering that there is a poor immune response to tumor associated antigens (TAAs) various strategies have been proposed to increase the immune response. Amongst them are new experimental options for treatment, for example, cytokine like IL-4, which facilitates an immune response against glioma[52] in a similar way to toll-like receptor (TLR) agonists. One example of this TLR agonist is Imiquimod, which could enhance T-cell responses to intracranial tumors, apart from reducing the number CD4(+)Foxp3(+) cells[53]. Costimulation of B7 molecule[54], blocks the B7-H1/PD-1 pathway with antagonistic antibodies to protect T cell responses[55].

Most immunotherapy attempts have had limited clinical success, with the exception of cellular immunotherapy using dendritic cell vaccines[56]. The multi-epitope-pulsed dendritic cell vaccine can be used for treatment. Dendritic cells are the most potent antigen-presenting cells for naive T cells, and can be obtained ex vivo from blood monocytes[57]. Monocytes are matured with the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, or IL-6, prostaglandin E2 (PGE2), IL-1β and the TNF-α[58], to obtain denditric cells. Mature DC (mDC) induces antigen-specific T-cell responses when mDC is pulsed with tumor lysate, cancer stem cells, or peptides from TAAs, as reported by Phuphanich *et al*[59]. These pulsed dendritic cells increase the immune response against tumor cells[59]. Amongst the various TAAs used for pulsed cells are antigens from gliomas or cancer stem cells which are HER2/N, TRP-2, AIM-2 or peptides.

It is more effective if multiple epitopes are used to target and enhance cancer vaccines[60]. The peptides used in the autologous vaccine mDC, could be a combination of peptides, for example, six synthetic class I peptides AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13Ra2. These were named ICT-107 and were selected from a glioma[58]. This combination of enhanced epitopes is clearly recognised by HLA class I -restricted T cells. This multi-epitope-pulsed dendritic cell vaccine can be administered intradermally at multiple sites.

In the treatment of patients with glioblastoma the use of many forms of therapeutic drugs could cause three main reactions, firstly increasing the brain edema which was a problem for the patient. Secondly, it is believed that brain tumor capillaries could limit the delivery of therapeutic drugs to the brain, and finally, the sum of many therapeutic drugs may easily lead an elderly patient into a delirious state.

There are many regulatory edema molecules in the brain. In the environment of the brain tumor, PGE2, aquaporins, aquaporin 1 (AQP1) and 4 (AQP4) exist. The glioma that infiltrate microglia are an important source of PGE2 and Cox-2, so Cox-2 inhibitors are proposed as an alternative to the use of glucocorticoids in peritumoral edema of malignant gliomas[15].

In short, in order to improve the quality of life in elderly patients with brain tumors, such as glioblastoma, many new treatment options should now be tested.

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EDITORIAL

Anti-cancer potential of litchi seed extract

Chih-Cheng Lin, Yuan-Chiang Chung, Chih-Ping Hsu

Chih-Cheng Lin, Department of Biotechnology, Yuanpei University, Hsinchu City 30015, Taiwan
Yuan-Chiang Chung, Department of Surgery, Cheng-Ching General Hospital, Taichung City 40764, Taiwan
Chih-Ping Hsu, Department of Medical Laboratory Science and Biotechnology, Yuanpei University, Hsinchu City 30015, Taiwan
Author contributions: Lin CC and Chung YC contributed equally to this article; Lin CC and Chung YC contributed to substantial contributions to conception and design, acquisition of data, and analysis and interpretation of data; Lin CC and Hsu CP contributed to drafting the article and revising it critically for important intellectual content; and Lin CC and Hsu CP gave final approval of the version to be published.
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Correspondence to: Chih-Ping Hsu, PhD, Department of Medical Laboratory Science and Biotechnology, Yuanpei University, No. 306 Yuanpei Street, Hsinchu City 30015, Taiwan. hsucp@mail.ypu.edu.tw
Telephone:+886-3-6108166 Fax: +886-3-6102312
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Abstract

Polyphenol-rich fruit are believed to be healthy food for humans. Traditional Chinese Medicines (TCMs) from fruit are rich sources of polyphenols and exhibit antioxidant and anti-inflammatory activities, and have been shown experimentally to overcome some chronic diseases, including cancer. The litchi seed is one of the TCMs traditionally used for relieving pain and sweating, and has been revealed in our recent report and other studies to possess rich amounts of polyphenolic species, including flavonoids and proanthocyanidines, and exhibits strong anti-oxidant activity, and could be applied for the treatment of diabetes and cancer. Herein, we review the recent findings regarding the benefits of this TCM in the treatment of human cancer and the possible cellular and molecular mechanisms of the litchi seed.

Key words: Litchi seed; Cancer; Cell cycle; Apoptosis; Traditional Chinese Medicine

Core tip: Litchi seeds possess rich amount of polyphenols and anti-cancer activity, which could be a potential cancer prevention or treatment agent.

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INTRODUCTION

Cancer is one of the most prevalent diseases worldwide, with high morbidity and mortality. It has been accepted that cancer is a progressive disease requiring slow and stepwise development for several years to become a life-threatening disease. Therefore, it is regarded largely as a preventable disease[1-3]. Recent advances in medical techniques have rendered some types of cancer curable, but other cancers are still difficult to cure, even under advanced treatment. Novel detection methods and treatment strategies must be developed[4]. Traditional Chinese Medicine (TCM) has been developed in China for more than two thousand years. TCMs comprise various forms of therapies, such as acupuncture, massage (Tui na), exercise (qigong), and dietary therapy, and the main part of these therapies is herbal medicines. A substantial amount of information from human, animal and cell line studies has provided evidence that consumption of certain herbal products used in TCM can exert chemopreventive effects[5]. Recent studies have revealed that some TCMs or their components exhibit anti-tumor activities towards several types of cancer, such as liver[6], lung[7], gastric[8], nasopharyngeal[9] and colorectal cancer[10]. Several clinically-used chemotherapeutic drugs are derived from TCMs, such as camptothecin, isolated from the "happy tree" (*Camptotheca accuminata*); etoposide, semi-synthesized from

a compound of *Podophyllum emodi var. chinense*; vincristin and vinblastin, isolated from the Madagascar periwinkle (*Catharanthus roseus*); and paclitaxel, purified from *Taxus chinensis*[11,12]. However, severe side effects and drug resistance always lead to therapy failure when using these chemotherapeutic drugs. Other types of substances need to be discovered to overcome these problems. Phenolic compounds have been accepted to be possible chemopreventive and treatment agents for cancer[13-16]. Polyphenols are obtained mainly from plants, and some have been regarded as forming part of a healthy diet for many years, such as tea, soybean, pomegranate, and pine nuts[17]. Litchi seeds have been analyzed and were found to possess rich amounts of polyphenols and exhibit strong antioxidant and inflammatory activities[18,19]. Recently, several studies by our research group and others have further revealed that litchi seed extract exhibits anti-cancer activity towards colorectal, liver, lung, and cervical cancer[19,20]. Herein, we review the recent findings regarding the benefits of this TCM in the treatment of human cancer and the possible cellular and molecular mechanisms of this substance.

LITCHI SEEDS IN TCM

The litchi (*Litchi chinensis*, Sapindaceae) is a tropical fruit tree that originates from southern China and is cultivated in semi-tropical areas world-wide for the delicious taste of the fruit[21]. A TCM pharmacopoeia named the *Compendium of Materia Medica* revealed that litchi seeds could be used to release or loose stagnant complexion, decadent colicky and the woman angry blood pain. Another TCM pharmacopoeia named *Ben-Cao-Yan-Yi* also recorded the analgesic effects of litchi seeds for heartache and intestinal pain. Yet another TCM pharmacopoeia named *Ben-Cao-Bei-Yao* described that the pharmacologic effect of litchi seeds could affect the liver and kidney and remove the stagnant humor, pathogenic cold and the woman angry blood pain. In Chinese folk remedies, Li-Ho-San, the mixture of litchi seeds, cumin and peel, can relieve the pain of a hernia or testicular swelling. Li-Shang-San, the mixture of litchi seeds and the root powder of *Aucklandia* lappa Decne., can treat gastralgia, period pain and postpartum abdominal pain. In summary, litchi seeds are used in China to release stagnant humor and remove chilling, and serve as an analgesic agent that can relieve the symptoms of coughing, gastralgia, neuralgia, and testicular swelling. However, scientific studies to prove the effects of the litchi seeds are still ongoing.

Evidence-based pharmacologic effects of litchi seed extract

In recent decades, several experimental studies have been performed in China on the pharmacologic effects of litchi seeds. Present pharmacological studies are mainly focused on the anti-hyperglycemic effect of litchi seeds. Pan and colleagues indicated that litchi seed extract or its components could repress blood sugar and liver glycogen in a rat non-insulin diabetes mellitus model[22]. Guo *et al*[23] reported that litchi seed water extract could increase insulin sensitivity and reduce the concentrations of blood fasting glucose, triglyceride, leptin and tumor-necrosis factor in a type-2 diabetes mellitus rat model. Li *et al*[24] revealed that litchi seed extract could decrease fasting blood glucose of alloxan induced diabetes mellitus rat to a level equal to that of normal rats. Indeed, many other Chinese reports have demonstrated that litchi seed extract can reduce hyperglycemia and restore the sensitivity to insulin in both type 1 or type 2 diabetes mellitus models. Litchi seeds also contain anti-hyperlipidemic agents. Pan and colleagues reviewed some Chinese studies and reported that litchi seed oil could prevent blood triglyceride and low density lipoprotein in a high-fat-fed rat model[22]. Zheng *et al*[25] revealed that litchi seed extract could inhibit the expression of the surface antigen of the hepatitis B virus. Zhang *et al*[26] found that litchi seed extract showed the protective effect in rat with nonalcoholic steatohepatitis, indicating litchi seed extract could overcome the liver damage from inflammation. In India, the seeds are powdered as an herbal medicine owing to their astringency, and after oral intake they have the reputation of relieving neuralgic pain[27]. These reports together indicated that the litchi seeds exert antihyperlipidemic, hypoglycemic and pain-relieving effects, implying multiple pharmacologic uses in TCM.

RECENT ADVANCES RELATED TO LITCHI FRUIT

Polyphenols in litchi and their pharmacologic effects

Recent studies have revealed that the litchi is a polyphenol-rich fruit. Litchi pericarp is composed of significant amounts of flavonoids and anthocyanins, including procyanidin B2, B4, epicatechin, cyanidin-3-retinoside, cyanidin-3-glucoside, quercetin-3-retinoside and quercetin-3-glucoside, *etc.*[27]. These components carry high free radical scavenging properties and could be used as anti-inflammation, anti-oxidation or anti-cancer agents[28,29]. Wang and colleagues showed that litchi pericarp ethanol extract inhibited the *in vitro* and *in vivo* growth of mouse hepatocellular carcinoma and both estrogen-dependent and -independent human breast carcinoma cells[30,31]. In recent reports, polyphenol compounds from litchi seeds were identified and found to be composed of a variety of proanthocyanidins and flavonoid glycoside[18,20,32]. Xu *et al*[32] revealed that litchi seeds contain litchitannin A1, litchitannin A2, aesculitannin A, epicatechin-(2βfOf7,4βf8)-epiafzelechin-(4Rf8)-epicatechin, proanthocyanidin A1, proanthocyanidin A2, proanthocyanidin A6, epicatechin-(7,8-bc)-4β-(4-hydroxyphenyl)-dihydro-2(3H)-pyranone, and epicatechin. All of these compounds exert strong anti-oxidant activity with ferric reducing antioxidant power values of 3.71-24.18 mmol/g and IC_{50} values of 5.25-20.07 μmol/L toward 2,2-diphenyl-1-picrylhydrazyl radicals. Litchitannin A2 exerts an anti-viral activity against coxsackie virus B3. Aesculitannin A and proanthocyanidin A2 exhibit anti-herpes simplex virus 1 activity[32]. The same research group also identified some flavonoid glycosides in the litchi seed,

Table 1 Sensitivity of various types of carcinoma cells to litchi seed extract (mean ± SD)

Cancer type	Cell line	IC50[1] (μg/mL)
Lung adenocarcinoma	A549	22.49 ± 1.02
Duke'C CRC	Colo 320DM	23.91 ± 2.25
Cervical carcinoma	C33A	24.45 ± 3.36
Duke'B CRC	SW480	26.33 ± 2.80
Oral carcinoma	SCC-25	36.80 ± 3.03
Breast carcinoma	MDA-MB-231	43.70 ± 2.76
Ovarian carcinoma	ES-2	45.46 ± 4.33
Lung large cell carcinoma	H661	52.47 ± 2.83

[1]Cells were cultured in complete medium and then treated with different concentrations of litchi seed extract at 37 ℃ for 24 h. Cells were trypsinized and the viable cells were counted using a hemocytometer under a microscope. The viability was calculated and the concentration with 50% viability was defined as the IC_{50}. CRC: Colorectal carcinoma.

including litchioside D, (-)-pinocembrin 7-*O*-neohesperidoside, (-)-pinocembrin 7-*O*-rutinoside, taxifolin 40-*O*-β-*D*-glucopyranoside, kaempferol 7-*O*-neohesperidoside, tamarixetin 3-*O*-rutinoside, and phlorizin[20]. Some of these compounds appear to exhibit anti-neoplasm activities in lung cancer, cervical cancer and hepatocellular carcinoma cells[20]. Another report from the same group also showed the anti-neoplastic activity of a cyclopropyl-containing fatty acid glucoside from the litchi seed[33]. In our report, rich amounts of flavonoids and condensed tannins [195.3 ± 6.7 and 230.2 ± 3.6 mg catechin equivalent/g of dry mass litchi seed extract (LCSP)] in LCSP were obtained by heating litchi seeds to 70 ℃ followed by 70% ethanol extraction[19]. The LCSP potently inhibits colorectal carcinoma (CRC) cell proliferation. According to these results, the litchi seed could be developed as a potent anti-tumor agent.

Anti-tumor activity of litchi seed: Over the last decade, the researchers were focused on litchi seed and its active components for the anti-tumor activity [34]. Chen and colleagues treated litchi seed water extract or granules to mouse xenograft of mouse Ehrlich ascites tumor cells, sarcoma S180 tumor cells and liver tumor cells and found the reduced tumors[35]. Chen and colleagues found that litchi seed could enhance both innate and acquired immunity in S180 cell xenograft[36]. Lv *et al*[37] demonstrated that litchi seed extract could reduce Bcl-2/Bax ratio in tumor tissues of sarcoma S180 mouse xenograft. Xu *et al*[20] isolated 7 different compounds from the litchi seeds and tested their cytotoxic activity towards human lung (A549), pulmonary (LAC), liver (Hep G2), and cervical (HeLa) cancer cell lines *in vitro* using the MTT colorimetric assay after 72 h. They found that kaempferol 7-*O*-neohesperidoside represented significant cytotoxicity towards all of the test cell lines, with IC_{50} values of 0.53, 7.93, 0.020 and 0.051 μmol/L, respectively. Litchioside D exhibited cytotoxic activity toward LAC and Hep-G2 cells (IC_{50} = 0.79 and 0.030 μmol/L). Taxifolin 40-*O*-β-*D*-glucopyranoside exerted cytotocic effects towards all four cell lines, with IC_{50} values ranging from 1.82 to 17.58 μmol/L. Compared with adriamycin, kaempferol 7-*O*-neohesperidoside represented more cytotoxic effect to these four cell lines[34]. Although the active components of litchi seeds against cancer have been revealed, Weber *et al*[38] suggested that the treatment approaches combined with an overall treatment protocol for the tumor microenvironment and chronic systemic inflammation are likely to provide a more successful outcome than a single tactical approach. According to these findings, they concluded that kaempferol 7-*O*-neohesperidoside, litchioside D and Taxifolin 40-*O*-β-*D*-glucopyranoside might be involved in the anti-tumor activity of litchi seeds.

Our recent report revealed that LCSP exhibits inhibitory effects on two colorectal cancer cell lines, SW480 and Colo 320DM[19]. Recently, we also tested the inhibitory effect of LCSP towards human lung adenocarcinoma cell line A549, lung large cell carcinoma cell line NCI-H661, cervical carcinoma cell line C33-A, breast carcinoma cell line MDA-MB-231, oral carcinoma cell line SCC-25, and ovarian carcinoma cell line ES-2, with IC_{50} values as shown in Table 1. The most sensitive cell lines were A549 cells, CRC cell line Colo 320DM, SW480 and C33A cells, with IC_{50} values of 22.49, 23.91, 26.33 and 24.45 μg/mL, respectively. SCC-25, MDA-MB-231, ES-2 and NCI-H661 were less sensitive towards LCSP treatment, with IC_{50} values of 36.8, 43.7, 45.46 and 52.47 μg/mL, respectively. These results further indicate the anti-neoplastic activity of the litchi seeds. However, the exact cellular and molecular mechanisms of LCSP or its components in the inhibitory effect of cancer cell growth require further investigation. Two possible mechanisms may be the induction of cell-cycle arrest and apoptosis. We reviewed recent evidence showing that LCSP could arrest cancer cells in the G_2/M phase and induce mitochondria-mediated apoptosis in CRC cells.

Possible mechanisms of the litchi seeds

LCSP arrests CRC cells in G_2/M: Our recent study revealed that LCSP-treated Colo 320DM and SW480 cell lines are partly arrested at the G_2/M phase. Cyclins are the key regulatory factors controlling the cell-cycle progression in cancer cells. According to our results, LCSP may disturb cyclin expression to arrest CRC cells at the G_2/M phase. Cyclin D1 is an important regulator of G1 phase progression in many different cell types, including CRC cells[39]. In our study, LCSP treatment decreased the level of cyclin D1 in Colo 320DM and SW480 cells, which was correlated with the cell cycle analysis showing G_2/M phase arrest. Moreover, disruption of cyclin A, a cyclin expressed during the S phase, can block DNA replication during the S phase[40]. Cyclin B is expressed in the G_2 and M phases of the cell cycle. A decrease in cyclin B blocks the cell cycle from progressing into mitosis[41]. Together with alteration of cyclin D1, these findings suggest that the effect of LCSP on the cell division cycle is mainly due to disturbance of G_2/M progression. Our previous studies demonstrated that flavonoids and proanthocyanidin-rich substances such as grape seeds, longan seeds or longan flower extract could increase the numbers of G_1- or S-phase cells in cancer cells[19,42-45].

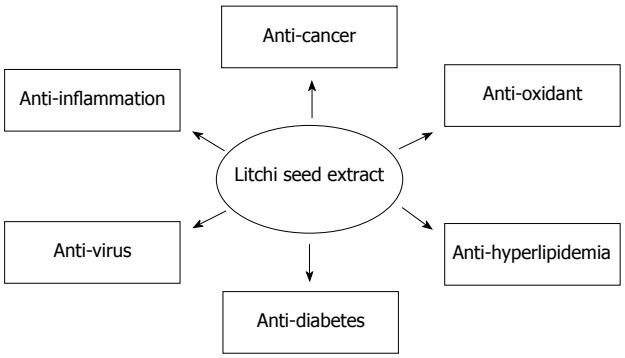

Figure 1 The multiple pharmacologic activities of litchi seeds on anti-cancer, anti-oxidant, anti-inflammation, anti-diabetes, anti-hyperlipidemia and anti-virus.

LCSP-treated CRC cells exhibited significant increases in the number of G_2/M-phase cells, which differed from previous reports. These findings suggested that the anti-proliferative effect induced by flavonoids and proanthocyanidin from naturally-occurring products could occur through a different cell-cycle-controlling mechanism. The different compositions of flavonoids and proanthocyanidin in each natural product might induce different expressions of cyclin proteins to control the cell cycle in CRC cells. Whether the alteration of cyclin D and A levels by LCSP treatment is the only molecular mechanism responsible for the perturbation of the M to G_1 phase of the cell cycle in CRC cells needs further investigation.

LCSP induces apoptosis toward CRC cells: Apoptosis is the elimination process to remove unwanted or damaged cells during development or maintenance of tissue homeostasis in multiple cellular organisms[46,47]. Dysfunction of apoptosis has been implicated as the main mechanism causing many human chronic diseases, such as neural degeneration, autoimmune disease, AIDS and cancer[48]. Many anti-cancer drugs and chemopreventive natural products possess activity to induce cancer cells into apoptosis and concomitantly suppress cancer cell growth[47]. In our recent study, we demonstrated that LCSP could induce CRC cells to undergo apoptosis[19]. The evidence came from the phosphatidylserine translocation to the outer leaflet of the plasma membrane, which was detected using annexin V analysis and activation of the caspase pathway in treated CRC cells. Caspase 3 expression and activation plays a crucial role in polyphenolic compound-induced apoptosis in CRC cells[42,44,49-51]. In our study, the active form of caspase 3 was increased in LCSP-treated CRC cells, further indicating that LCSP-induced apoptosis is mediated by caspase 3 activation. The subsequent increase in cleavage of caspase 3 substrate PARP in LCSP-treated CRC cells confirmed the activation of caspase 3. Involvement of the Bcl-2 family of proteins may play an important role in LCSP-induced apoptosis. The Bcl-2 family members are important mediators of mitochondria-induced apoptosis in cancer cells[46,52,53]. These proteins form multimers, which act as pores in cell membranes, controlling the flow of molecules[54]. Bcl-2 proteins are important mediators of apoptosis in CRC cells[46,47]. Some family members promote apoptosis (*e.g.*, Bax and Bad), while others inhibit it (*e.g.*, Bcl-2 and Bcl-x)[55,56]. Bcl-2 inhibits apoptosis by inhibiting the release of cytochrome c (Apaf 2) and apoptosis inducing factor from the mitochondria to the cytoplasm, and by limiting the activation of caspase 3 by inhibiting its activator protein, Apaf 1[57]. Some studies have suggested that the ratio of Bax:Bcl-2 proteins is the determining factor in transmission of the apoptotic signal[54,58-60]. Previously, proanthocyanidine-rich grape seed extract has been found to suppress the expression of Bcl-2 protein in breast and skin carcinoma cells[61,62]. Additionally, in our previous reports, we also confirmed that longan seed extract increases the Bax:Bcl-2 ratio in CRC cells[44,63]. The Bax:Bcl-2 ratio in LCSP-treated CRC cells increased significantly, indicating the importance of the Bax:Bcl-2 ratio in cancer cell life and death[54,58]. Taken together, our results demonstrated that LCSP-induced apoptosis in CRC cells was mediated by an increasing Bax:Bcl-2 ratio, by which LCSP induced mitochondria-mediated apoptosis in CRC cells. Although the anti-cancer activity of Litchi seed extract has been revealed, the toxicity to normal cells and the possible side effect has not yet been studied. Wan and his coworkers found that oral administration of the maximum dosage of litchi seed water or ethanol extract could not cause acute toxicity to mouse[64]. However, in our recent unpublished result, litchi seed extract exhibited suppression effect on normal small intestinal cells and lung fibroblast cells at more than 50 μg/mL. These results implicated the usage of litchi seed extract at lower dose and the possible toxicity may occur in gastrointestinal and lung system.

CONCLUSION

The litchi is one of the most important fruits in China, economically speaking. The seeds of the litchi were regarded as waste for a long time, and failed to be utilized. However, according to TCM pharmacopoeia, litchi seeds possess multiple pharmaceutical applications. Recent advanced biotechnology and pharmacology techniques have allowed us to gain deeper insight into the functions of this TCM using scientific methods. Litchi seed extract could overcome metabolic diseases such as diabetes mellitus, decrease triglycerides and suppress oxidation and inflammation. Some components of the litchi seed have been identified to be anti-cancer agents against lung, liver, pulmonary and cervical cancer. We further provide data to demonstrate that LCSP is also capable of inhibiting the growth of colorectal carcinoma, lung adenocarcinoma, lung large cell carcinoma, breast carcinoma, oral carcinoma, cervical carcinoma, and ovarian carcinoma cells. All of the pharmacologic effects of litchi seed extract are summarized in Figure 1. The main mechanisms of LCSP are the induction of cell-cycle arrest and apoptosis, at least in colorectal cancer cells, with the molecular mechanisms acting through decreased levels of cyclin D1, A and B1 and alteration of the Bax:Bcl-2 ratio and

activation of caspase 3. However, upstream factors mediating LCSP induction of cell-cycle arrest and apoptosis need further investigation. We found that LCSP treatment could inhibit proliferation in various cancer cells and induce cell-cycle arrest and apoptosis in CRC cells, suggesting its potential as a novel chemoprevention agent for cancer in the future.

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REVIEW

RNA vaccines for anti-tumor therapy

Fabian Benencia

Fabian Benencia, Biomedical Engineering Program, Russ College of Engineering and Technology, Ohio University, Athens, OH 45701, United States
Fabian Benencia, Molecular and Cell Biology Program, Ohio University, Athens, OH 45701, United States
Fabian Benencia, Department of Biomedical Sciences, Heritage College of Osteopathic Medicine Academic Research Center, Ohio University, Athens, OH 45701, United States
Author contributions: Benencia F solely contributed to this paper.
Supported by Ohio University and a Research Scholarly Affairs Committee grant award to Benencia F, No. RP1206
Correspondence to: Fabian Benencia, PhD, Assistant Professor of Immunology, Department of Biomedical Sciences, Heritage College of Osteopathic Medicine Academic Research Center, Ohio University, 61 Oxbow Trail, Athens, OH 45701, United States. benencia@ohio.edu
Telephone: +1-740-5972133 Fax: +1-740-5972778
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Abstract

The immune system is able to recognize tumor antigens and this has been the basis for the development of cancer immunotherapies. The immune system can be instructed to recognize and attack tumor cells by means of vaccination strategies. One such strategy involves the delivery of tumor antigen as genetic material. Herewith we describe the use of RNA encoding tumor antigens for vaccination purposes in tumor settings. RNA has features that are interesting for vaccination. Upon transfection, the RNA has no possibility of integration into the genome, and the tumor translated proteins enter the intrinsic antigen processing pathway thus enabling presentation by MHC-I molecules. This can specifically activate cytotoxic CD8 T cells that can attack and kill tumor cells. RNA can be delivered as a naked molecule for vaccination purposes or can be used to transfect dendritic cells. The combination of RNA technology with dendritic cell vaccination provides a powerful tool for cancer immunotherapies.

Key words: RNA; Vaccine; Dendritic cells; Immunization; Cytotoxic T cells; Tumors

Core tip: In this review we discuss the use of RNA encoding tumor antigens for anti-tumor vaccination. RNA has several features that makes it relevant for vaccination purposes. Importantly, the RNA has no possibility of integration into the genome, and the tumor translated proteins enter the intrinsic antigen processing pathway thus enabling presentation by MHC-I molecules thus specifically activating cytotoxic CD8 T. Further, RNA can be delivered as a naked molecule f or can be used to transfect dendritic cells. This combination of RNA technology with dendritic cell vaccination provides a powerful tool for cancer immunotherapies.

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TUMOR IMMUNOLOGY

Cancer is one of the leading causes of mortality in humans and most of the successes obtained battling this disease rely on early prevention even though a gamut of treatments such as chemotherapy, radiotherapy and surgery are available to patients. In view of this situation it becomes necessary to generate innovative approaches for the treatment of this disease. One such strategy entails educating the immune system to recognize and destroy tumor cells. To this end, several immunotherapeutic strategies have been designed and tested in preclinical studies and clinical trials.

Tumors are composed not only by cancer cells, but also by other cellular types such as fibroblasts, endothelial

cells and infiltrating leukocytes that together with extracellular matrix components constitute the microenvironment of the tumor[1]. In recent years the relevance of the tumor microenvironment as a key player in cancer progression has been highlighted and the role of its cellular populations and extracellular matrix components examined. In this context, immune cells play a double edge sword role[2].

On one hand, the protective role of the immune system against tumors has been widely described and indeed the presence of tumor-infiltrating lymphocytes (TILs) has been reported in numerous studies involving melanoma[3], colorectal[4-7], breast[8,9], ovarian[10-16], prostate[17], renal[18], and esophageal carcinoma[19]. These TILs are able to recognize tumors as demonstrated by their capability to get activated by tumor antigens and kill cancer cells *ex vivo*[10,20-22]. Notably, several reports showed that the prevalence of certain T cell populations is associated with a better outcome in different types of cancers. Particularly, studies involving ovarian, non-small cell lung, mesothelioma, colon, and urothelial cancers showed that a high CD8/regulatory T cell ratio among TILs is usually associated with a better prognostic or a better response to antitumor treatment[14,23-28].

On the other hand, the presence of a robust number of regulatory T cells within the TILs, or a CD4/CD8 ratio that favors CD4 T cells, has been associated with a worse outcome or tumor growth in various studies[29-33]. These studies highlight the ability of the immune system to recognize tumors and provide a rationale for pursuing immunotherapeutic approaches, but also underscore the hurdles for its success. Similarly, other tumor-associated leukocytes such as myeloid-derived suppressor cells (MDSCs) can promote tumor growth by modulating the immune response[34]. Indeed, we have previously demonstrated the relevance of the tumor microenvironment in attracting MDSCs by a complement-mediated process[35]. Further, the presence of a subset of splenic dendritic cells (DCs) with the ability to suppress antitumor T cells responses *via* indoleamine 2,3-dioxigenase expression highlights the immunosuppressive role of antigen presenting cells (APCs) in some tumor settings[36]. Notably, leukocyte infiltration can precede the development of a neoplasm, being chronic inflammation a risk factor for the development of cancer[37-39]. Further, inflammatory conditions such as caused by certain types of infections can be involved in the pathogenesis of many human malignancies. For example, gastric carcinomas can arise in a *Helicobacter pylori*-induced gastritis environment[38] or hepatitis B virus/hepatitis C virus can induce hepatocellular carcinomas[39]. Also, chronic but non-infective inflammatory conditions as in the case of smoking-related bronchial cancer can induce carcinogenesis[40]. In the same way, chronic pancreatitis is considered a risk factor for the development of pancreatic cancer, and many of the growth factors involved in tissue remodeling and regeneration in chronic pancreatitis are present in pancreatic cancer[41]. In addition, there is strong evidence that tumor-associated leukocytes can also promote tumor angiogenesis. In particular, infiltrating inflammatory cells secrete a diverse repertoire of growth factors and proteases that potentiate tumor growth by stimulating angiogenesis. We and others have described the capability of APCs such as DCs or macrophages, to collaborate with neoangiogenesis in human cancers and in different mouse tumor models[42-47]. Thus, tumors exhibit an arsenal of mechanisms in order to inhibit an effective immune response.

Collectively, these data indicate that in some settings immunoablative procedures must precede immunotherapeutic treatments. To this end, some studies have suggested that depletion of regulatory T cell populations or tumor-associated leukocytes can enhance the effectiveness of a subsequent immunotherapy[3,48].

TUMOR IMMUNOTHERAPY

The ability of the immune system to recognize and attack tumors relies on the presence of tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). As recently reviewed by Aly[49], TSAs are expressed only by tumor cells due to mutations in normal cellular genes, or to the expression of viral antigens or normally suppressed oncogenes in cancer cells. On the other hand, TAAs are molecules expressed both in normal and cancer cells but expressed at higher levels by tumors, or expressed by normal cells only during the embryonic state differentiation. For the purposes of the present review, tumor antigens will be named generically as TAAs.

Pioneering studies performed by Rosenberg *et al*[50] in melanoma aimed to activate lymphocytes *in vivo* by treating cancer patients with IL-2. The rationale being that the patients' T cells have the ability to recognize and attack tumors. Indeed, this is the basis for immunotherapies using TILs. To carry out these T cell adoptive therapies, upon purification from tumor tissues, TILs are expanded and activated *ex vivo* using TAAs and are subsequently re-infused into patients[51]. Recent advances in this area involve the generation of TAA-specific T cells by means of genetic recombination. As previously described in detail, chimeric antigen receptor (CAR) T cells are engineered to express the portion of an antibody that recognizes an antigen fused to the T cell receptor signaling region[52]. Thus, they recognize TAA on tumor cells with the specificity of an antibody and they kill them using the cytotoxic machinery of T cells[52]. This circumvents the problem of isolating TILs, which might not be present in all patients or present at very low numbers in tumor samples. Recently, by using CAR T cells, Kalos *et al*[53] were able to completely eradicate cancer cells in patients with advanced leukemia.

Additional immunotherapeutic strategies have been proposed and investigated based on the ability of the immune system to recognize TAAs. One such strategy involves inducing immune responses against TAA by means of vaccination. To this end, TAAs are used as tumor lysates, proteins purified from these lysates, or pep-

tides (derived from tumor protein digests or synthesized *in vitro*). Furthermore, as described below, tumor vaccination strategies also involve the use of apoptotic or necrotic tumor cells as way of delivering the TAAs. These molecules will be recognized *in vivo* by resident APCs, which are key components of the innate immune system. The innate immunity is the first line of defense against pathogens. Cells of the innate immune response include macrophages, granulocytes, DCs, and natural killer cells. Macrophages, B lymphocytes and DCs are generally described as APCs. After ingesting a pathogen, APCs are able to eliminate it through various mechanisms involving enzymatic degradation and the use of reactive oxygen or nitrogen species. APCs detect pathogens through the expression of pattern recognition receptors (PRRs) which are able to recognize conserved pathogen associated molecular patterns (PAMPs). Some of the main PRRs include membrane associated toll-like receptors (TLRs) and cytoplasmic NOD-like receptors[54,55]. DCs are highly effective APCs distributed throughout the body, particularly in immunological organs such as thymus, spleen, lymph nodes and Peyer's patches[56-58].

DC ACTIVATION PROCESS

Immature (non-activated) DCs present in peripheral tissues can detect PAMP-bearing microorganisms through their high expression of cell surface, vesicular and cytoplasmic PRRs[59]. This process leads to the activation of the DCs, which can degrade pathogenic proteins (both recovered from the extracellular space, or from the cytoplasmic pool) and process them into peptides[58]. Antigenic peptide fragments derived from the processed pathogen molecules are the exposed on the surface of the DCs in the context of MHC I or Ⅱ molecules. During this process, an immature DC will undergo "maturation" due to presence of inflammatory cytokines generated by the DC itself, or by other surrounding cells in response to the pathogen or tissular damage. This maturation process entails upregulation of MHC class Ⅱ molecules, costimulatory molecules such as CD40, CD80, CD86; OX40L and the chemokine receptor CCR7. This receptor recognizes the chemokines CCL19 and CCL21 which are constitutively expressed at high levels by lymph nodes[60]. Thus, mature DCs migrate from the sites of antigen capture to the T-cell regions of draining lymph nodes, where they contact naïve or memory T cells. Through interaction with specific cell receptors for antigen on the surface of T lymphocytes, DCs select and activate specific T cell clones with the capability to recognize the presented antigen[58,61,62]. In this way, DCs tie the innate and adaptive immunity, being keystones for the development of antigen specific immune responses.

APCs have different ways of processing and presenting antigens. Typically, antigens that are captured by the phagocytosis or endocytosis are degraded in the lysosomal compartment and peptides are presented by MHC-Ⅱ molecules on the surface of the cells thus interacting and activating CD4 T cells. On the other hand, antigens generated within the cells for example as a result of a viral infection, can be degraded by the proteosome and the peptides presented on the surface of the cell in the context of MHC I molecules[55,61,63]. This strategy selects and activates antigen specific CD8 T cells[55, 61,63]. Notably, DCs have the capability to cross-present antigens[64]. This means that DCs can acquire extracellular antigens, like for example apoptotic or necrotic tumor cells, or tumor lysates and also present them to CD8 T cells in the context of MHC I molecules.

DCS AND ANTI-TUMOR THERAPY

A multitude of preclinical studies and clinical trials have been designed in order to determine the anti-tumor efficacy and safety of DC-based vaccines[65]. The development of a successful DC-based tumor vaccination depends heavily on generating robust and long lasting specific CD4 and CD8 T cell responses[66]. To accomplish this, DCs have been generated from bone marrow precursors in the mouse and mostly from monocytes in humans as we previously reviewed[1]. Different steps in the antigen presentation process have been evaluated such as antigen loading, DC maturation, and delivery route and dose scheme as we have recently reviewed[1]. One strategy for loading DCs with TAAs in the mouse model involves pulsing the cells with peptides derived from tumor antigens[67]. In addition, since TAAs are not well characterized for the majority of tumors, vaccines can be prepared with whole tumor antigens[68,69]. To this end, DCs have been loaded with whole tumor lysates[70], apoptotic or necrotic cells[71] alone or conjugated with TLR ligands[72], antigens coated with antibodies to target them to Fcγ receptors[73] or peptides encapsulated in biodegradable polymers[74]. We have showed that inducing the expression of danger signals in tumor cells by means of replication-deficient or replication-restricted virus appears also to be an efficient method to pulse DCs for vaccination purposes, probably by upregulating danger signals in the tumor cells[71]. Finally, other strategies such as fusing DCs with tumor cells have also been successfully pursued[75]. These fused cells express tumor antigen but had the machinery of the DCs to present these antigens to T cells.

This information regarding DC-based antitumor vaccines pulsing has been translated to the human, where clinical trials have involved, among others, DCs pulsed with peptides[76], whole tumor lysates[77], or fused with tumor cells[78-80]. Other strategies involved pulsing human DCs with apoptotic or necrotic cells[81-90]. As we have previously reviewed[91] controversy exists regarding whether necrotic or apoptotic cells are better for pulsing DCs for tumor vaccination purposes[90,92-94]. Nevertheless, inducing tumor cell death by exposure to ultraviolet-B radiation seems to provide a mixture of apoptotic and necrotic cells suitable for vaccination purposes DCs[95,96].

TAA AS GENETIC MATERIAL

Another vaccination strategy entails delivering TAAs as

the genetic material that encodes their synthesis. Thus, either DNA or RNA carrying the information to synthesize TAAs can be administered to laboratory animals in preclinical studies or to patients under clinical trials with the aim to induce local synthesis of TAAs. In contrast to delivery of TAAs as protein/peptide formulations, the recombinant antigens synthesized in the cytosol of the cells may enter the degradation process of intracellular molecules, yielding peptides that can be directly presented by MHC I molecules hence inducing a robust CD8 (cytotoxic) T cell immune response. To this end, numerous studies have been performed in order to determine the effectiveness of DNA vaccination in tumor settings[49,97,98]. The genetic material can be administered *in vivo* by using different techniques such gene gun, ultrasound, electroporation, cationic liposomes, and nanoparticles[99]. Alternatively, viral vectors can deliver DNA encoding for TAAs directly to the DCs. Viral vectors used to transduce human DCs[100] include recombinant adenoviruses[101-103], poxviruses[104], and retrovirus[100]. Lentiviruses have also been used to induce stable transduction of human hematopoietic stem cells or DCs[105,106]. These vectors have the advantage of infecting non-dividing cells, therefore being excellent tools to express different molecules in terminally differentiated DCs which have lost the capability to duplicate. Moreover, hematopoietic stem cells have been transduced with lentiviruses and then differentiated into antigen-expressing DCs[107]. The full scope of DNA vaccination has been extensively reviewed in the literature and will not be discussed here.

RNA VACCINES

An alternative approach for delivering TAAs as genetic material is the use of RNA for vaccinations. The advantage of RNA vaccination in comparison to DNA vaccination is that there is no danger of genome integration with the latent possibility of oncogene activation, and that there is no need to engineer expression vectors for delivery. On the contrary the expression of the antigens in the context of RNA delivery is transient, and then RNA is very labile as compared to DNA. Both DNA and RNA vaccines in addition to carrying TAAs have the potential to non-specifically stimulate the immune response upon recognition of CPG sequences by TLR9 (DNA) or by activation of TLR3 (RNA). RNA vaccination strategies involve naked RNA delivery or the pulsing of DCs with RNA molecules. Further, both whole tumor RNA or TAA specific RNA have been used as inducers of antitumor immunity.

VACCINES WITH NAKED RNA

Murine studies

Several murine studies describe the use of naked RNA for vaccination purposes. The naked RNA can be administered by injection or delivered intradermally through electroporation[108]. In order to decrease degradation, the RNA has been complexed with histidine-rich cationic polymers and histidylated cationic lipids. In this case, systemic injections of specific synthetic messenger(m) RNA encoding the human melanoma MART-1 TAA complexed with polyethylene glycol ylated histidine-rich polylysine and histidylated liposomes (termed lipopolyplexes) were able to delay the growth of B16F10 melanoma in the mouse model[109]. Notably, intravenous injection of mannosylated liposomes containing mRNA encoding for the EGFP protein proved to be taken up by spleen DCs. Further, when mRNA for MART-1 was complexed into these mannosylated liposomes, a decrease in the growth of B6F10 murine melanoma tumors was observed[110].

Another strategy is to deliver naked RNA that could simultaneously activate the immune response by way of TLR signaling. These kind of vaccines are called "two component" since they deliver TAAs while simultaneously activating the immune response. It has been reported that two component OVA-encoding RNA vaccines containing free and protamine-complexed mRNA induced specific immune responses activating both humoral and cellular immune responses against OVA-expressing tumors[111]. In addition, naked RNA can be injected systemically, or can be administered directly to sites harboring high concentration of immune cells by means of intranodal injection[112,113]. This strategy aims to directly target APCs in the site where they interact with T cells.

An innovative approach to RNA vaccine immunotherapy has been the developing of self-replicating RNA vectors (replicons). These vectors encode for a RNA-dependent RNA polymerase derived from alphaviruses which has the capability to amplify a plasmid-encoded TAA RNA[114]. This increases the availability of TAA RNA and consequently, TAA protein availability. In addition, this counteracts the high degradation that naked RNA is subjected to upon injection. Immunization with RNA replicons encoding for HPV antigens was able to decrease the growth of aggressive TC1 tumors, which carry HPV E6 and E7 antigens[115].

Human studies

Naked RNA vaccinations have been assayed in clinical settings. In particular, naked RNA encoding for several TAAs has been delivered intradermally inducing expression of cytotoxic T cells in cancer patients, together with an improve on the clinical response in some individuals[116,117]. In order to enhance the effectiveness of the transfection process while protecting the RNA from degradation, naked RNA has also been delivered complexed with liposomes in human clinical studies[118]. Further, both in mouse and human studies, adjuvants that target APCs such as FLT3 and GM-CSF have been co-delivered in their protein state or as RNA together with the naked RNA vaccines in order to further activate these cells locally[118]. This strategy aims to induce a robust activation of the transfected DCs *in vivo*, thus potentiating their migratory potential and their ability to induce the activation of T cells capable of recognizing TAAs of interest.

USE OF RNA-PULSED DCS FOR ANTITUMOR THERAPIES

Mouse studies

Foundational studies evaluating the effectiveness of DC-based RNA vaccination in the mouse model and in humans were performed by Dr Eli Gilboa. In 1996 his group was able to demonstrate that murine DCs pulsed with whole tumor RNA were able to induce a robust antitumor immune response in a mouse model of melanoma[119]. Shortly after, they were able to demonstrate the feasibility of this approach in a preclinical setting, inducing specific T cell responses *in vitro* by pulsing human monocyte-derived DCs with the carcinoembryonic antigen (CEA) antigen[120]. Since then, a multitude of studies have built on these successes in order to generate efficient DC-based RNA vaccines.

In animal experimental models, the efficacy of RNA-pulsed DC vaccination has been extensively tested. Collectively, vaccinated animals showed a decrease in tumor growth together with the activation of tumor specific cell-mediated immunity. In particular, murine DCs have been pulsed with whole tumor RNA as a source of TAAs[121-125]. Interestingly, we have previously reported that DCs pulsed with whole tumor RNA are more effective in inducing antitumor immune responses than DCs loaded with equivalent amounts of apoptotic tumor cells[126]. In order to enhance antigen presentation by DCs and the consequent efficacy of the vaccination procedure, DCs have also been pulsed with specific TAA mRNA replicons[127]. As described above, these constructs aim to increase the amount of TAA RNA present in the APCs with the consequent increase in the levels of expression of the antigen.

Other strategies designed to increase the effectiveness of DC-based RNA vaccination entailed pulsing DCs with TAA mRNA together with mRNA of cytokines such as GM-CSF and particularly IL-12[128-131], the rationale being that these cytokines will potentiate the degree of activation of the pulsed DCs.

Alternative strategies focused on enhancing the processing of the nascent TAA in the transfected DCs. To this end, studies pulsing DCs with RNA encoding for TAAs fused with molecules that augment the delivery of the synthesized proteins to the endoplasmic reticulum, TAAs RNA linked with ubiquitin RNA to target the ubiquitin-proteosome pathway, MHC I and Ⅱ pathways by fusion with LAMP1 or DC. LAMP sequences, or with immunogenic helper proteins such as EGFP have been used[97,132-134]. In this way, cytoplasmic TAAs will be more efficiently processed by the ER, increasing the levels of TAAs peptides presented in the context of MHC I molecules on the surface of the DCs.

Finally, others strategies to potentiate the efficacy of DC-based RNA vaccines entail the use of different maturation cocktails or immunostimulatory factors to activate the RNA-pulsed cells. For example, soluble CD40 has been shown to act as an adjuvant for cytokine treatment of RNA-pulsed DCs increasing the generation of cytotoxic T cells in a an experimental model of melanoma[135].

Human preclinical

In order to optimize the likelihood of effective translation into the clinic, human DCs have been prepared from monocytes recovered from apheresis products or by differentiation of $CD34^+$ hematopoietic precursors[136,137]. As above, whole tumor RNA or mRNA can be used to transfect these cells by electroporation or lipofection[137,138]. In addition, RNA recovered from tumor cells lines can be used to pulse human DCs. For example, whole RNA from KL562 leukemia cells was delivered to monocyte-derived DCs by electroporation and lipofection being the transfected RNA degraded within 24 h. Notably, the translated TAA proved to be processed through the MHC-I presentation pathway rather than the endosomal-phagocytic pathway indicating that these DCs could be able to activate CD8 cytotoxic T cells[139]. Interestingly, not only monocyte or hematopoietic $CD34^+$ derived DCs have been tested in RNA vaccination studies. Indeed, DCs directly recovered from hepatocellular carcinoma patients could be efficiently pulsed with whole RNA recovered from hepatic cancer cell lines[140].

It has been determined that better expression of TAAs after transfection with whole tumor RNA is achieved when antisense RNAs are eliminated from the whole tumor RNA preparation[141]. This highlights the need to prepare high quality RNA for transfection studies. Further, although most of DC protocols (both in mouse and human) propose to induce maturation of these cells after RNA transfection, a study suggests that RNA transfection of DCs can also be performed after maturation of these cells[142]. Taking into account studies indicating the viability of cryopreserved mature human DCs[143], this opens the possibility of transfecting DCs right before administration to patients.

Human DCs transfected with tumor RNA have been shown to elicit specific T cell responses *in vitro*. This was demonstrated by their ability to generate TAA specific T cell lines, or by activating *ex vivo* TILs recovered from cancer tissues. For example, DCs transfected with survivin or TERT RNA were used to generate CD8 cytotoxic cell lines with the capability to eliminate tumor cell lines and primary tumors *in vitro*[144,145]. Further, RNA recovered from prostate tumor samples by laser capture microdissection was amplified and used to transfect DCs generated from blood precursors. It was shown that these DCs were able to induce cytotoxic T cells *in vitro*[146].

As described above, mouse studies determined that RNA encoding for TAAs can be engineered to enhance the capability of the DCs to process the nascent antigens. To translate these results into the human setting, DCs generated from human monocytes were transfected with mRNA encoding for the TERT antigen fused with LAMP in order to augment the processing of the TAA upon translation. This strategy induced a robust activation of CD4 T cells specific for TERT as determined in

in vitro studies[147].

Human clinical studies

Several clinical trials have been conducted in order to evaluate the efficacy of DC-based RNA vaccines in cancer patients. In these clinical trials, the vaccines were generated by pulsing monocyte-derived DCs either with whole tumor RNA or specific TAA RNA. Altogether, human clinical studies highlight that the administration of DC-based RNA vaccines is safe and does not induce adverse reactions. For example, in a phase I clinical trial involving acute myeloid patients aiming to generate clinical grade DC vaccines, monocyte-derived DCs were pulsed with *in vitro* transcribed RNA encoding the Wilm's tumor. Then, these cells were injected repeatedly into patients by the intramuscular route[148]. The results of this study indicated that the vaccination scheme was well tolerated by the patients[148]. This was also observed in a clinical trial involving stage IV malignant melanoma patient[149]. In this case, DCs were pulsed with whole tumor RNA expanded *in vitro* but no positive effect of the vaccination was observed. This is no surprising taking into account the advanced stage of the illness, but nevertheless the study highlights the safety of using this procedure for antitumor therapies. Other studies showed that DC-based RNA vaccination is able to induce specific T cell responses in cancer patients. In particular, in a clinical trial involving relapsed metastatic ovarian cancer patients, DCs pulsed with mRNA specific for folate receptor α were able to induce a large population of effector memory CD8 cytolytic T cells reactive to the antigen upon repeated injections[150]. Similarly, specific T cell responses were observed in colorectal cancer patients receiving several injections of DCs harboring CEA mRNA[151]. In addition, it has been shown that patients vaccinated with DCs transfected with mRNA recovered from autologous melanoma tumor cells were capable of initiating T cell responses specific to antigens encoded by the pulsed APCs[152]. Finally, in order to ensure a robust activation of T cells, strategies designed to deliver the transfected DCs directly to the lymph nodes have been tested. In a phase Ⅰ/Ⅱ clinical trial with melanoma patients it has been shown that upon intranodal administration, DCs electroporated with mRNA encoding for gp100 or tyrosinase migrate towards T cells areas of the lymph node [153].

CONCLUSION

In closing, in the last 15 years, a growing body of literature has argued for the use of RNA for vaccination purposes. Importantly, RNA is safer than DNA vaccine approaches taking into account that no possibility of genomic integration exists. Furthermore, the combination of RNA technology with DC-based vaccines has made available a powerful strategy for antitumor therapies. Advances in RNA technology (*i.e.*, strategies to increase stability, use of replicons), together with the development of more effective protocols for generating activated DCs (*i.e.*, use of better inflammatory cocktails) and an increase in our knowledge of tumor immunology (*i.e.*, the use of immunoablative therapies to eliminate suppressor populations) will guide further pursuit of tumor immunotherapies using DC-based RNA vaccines. This offers the potential to advance the outcome of cancer immunotherapies for the benefit of patients.

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REVIEW

Mechanotransduction in bone: Intervening in health and disease

Anastasia Spyropoulou, Efthimia K Basdra

Anastasia Spyropoulou, Efthimia K Basdra, Department of Biological Chemistry, Cellular and Molecular Biomechanics Unit, University of Athens Medical School, GR-11527 Athens, Greece
Author contributions: Spyropoulou A and Basdra EK contributed to this paper equally.
Correspondence to: Efthimia K Basdra, PhD, Associate Professor, Department of Biological Chemistry, Cellular and Molecular Biomechanics Unit, University of Athens Medical School, 75, M. Asias Street, GR-11527 Athens, Greece. tegk.84@gmail.com
Telephone: +30-210-7462547 Fax:+30-210-7791207
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Abstract

Mechanotransduction has been proven to be one of the most significant variables in bone remodeling and its alterations have been shown to result in a variety of bone diseases. Osteoporosis, Paget's disease, orthopedic disorders, osteopetrosis as well as hyperparathyroidism and hyperthyroidism all comprise conditions which have been linked with deregulated bone remodeling. Although the significance of mechanotransduction for bone health and disease is unquestionable, the mechanisms behind this important process have not been fully understood. This review will discuss the molecules that have been found to be implicated in mechanotransduction, as well as the mechanisms underlying bone health and disease, emphasizing on what is already known as well as new molecules potentially taking part in conveying mechanical signals from the cell surface towards the nucleus under physiological or pathologic conditions. It will also focus on the model systems currently used in mechanotransduction studies, like osteoblast-like cells as well as three-dimensional constructs and their applications among others. It will also examine the role of mechanostimulatory techniques in preventing and treating bone degenerative diseases and consider their applications in osteoporosis, craniofacial development, skeletal deregulations, fracture treatment, neurologic injuries following stroke or spinal cord injury, dentistry, hearing problems and bone implant integration in the near future.

Key words: Mechanotransduction; Bone remodeling; Bone disease; Bone health

Core tip: Mechanotransduction has been shown to be of major significance in modulating bone remodeling under physiological and pathological conditions. Therefore the study of the underlying mechanisms is of major importance and necessary step towards the better understanding of bone biology as well as the development of therapeutic strategies against conditions characterised by deregulated mechanotransduction. This review will consider the molecular mechanisms behind mechanotransduction as well as the scientific models currently used for its better understanding. It will also focus on mechanostimulatory techniques that could be used against a variety of deregulated mechanotransduction-related diseases.

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INTRODUCTION

Bone tissue biology

The importance of bones for a living organism is undeniable and goes far from just providing structural support for the body, protecting vital organs and exchanging minerals. Bones also comprise a multi-functional system that

interacts with other systems and abnormalities in bone tissues may result in mild or severe diseases.

Bone tissue is composed of the bone matrix and five different cell types. The bone matrix contains an inorganic (carbonated hydroxyapatite) and an organic phase (mainly type I collagen and several growth factors) whilst the cellular content of the bone tissue comprises of osteoprogenitors, osteoblasts, osteocytes, osteoclasts and lining cells[1]. Osteoprogenitor cells comprise pluripotent cells of mesenchymal origin, localised on bone surfaces[1] which have the ability, under the appropriate conditions, to commit and differentiate towards osteoblasts[1]. On the same bone osteoblasts, the bone forming cells, are cited. They are responsible for the protein synthesis of the bone matrix as well as its calcification[1]. The cavities of the calcified bone matrix bear osteocytes which comprise entrapped inactive osteoblasts forming a net of communicating cells inside the calcified matrix[1]. Osteoclasts are large multinucleate cells of blood monocyte origin, settled inside bone resorption lacunae and they are responsible for bone resorption in bone remodeling areas[1]. Lining cells comprise inactive osteoblasts with the ability to protect bone surfaces from bone resorption[1].

Runt-related transcription factor 2 transcription factor in bone biology

Runt-related transcription factor 2 (Runx2) or core-binding factor subunit alpha-1 (Cbfα1), the major osteo-specific transcription factor[2] is responsible for the regulation of osteoblast differentiation as well as for hypertrophic cartilage synthesis[2,3]. Its expression is necessary and sufficient for the commitment of mesenchymal cells towards the osteoblastic cell line[4].

Abnormalities in Runx2 expression are indicative of its importance in bone biology. When Runx2 is expressed ectopically it has been shown to lead to increased expression of osteocalcin, alkaline phosphatase, collagenase-3, bone sialoprotein and collagen type Iα1[5]. Osteoblast maturation in mice bearing a mutant *runx2* gene is inhibited and thus so are the procedures of intramembranous and endochondral ossification[6,7]. Furthermore, it has been shown that differentiation of stem cells in adipocytes and chondrocytes in *runx2* knockout mice has not been impaired. In addition, heterozygous mice (*runx2*$^{-/+}$) developed characteristic skeletal abnormalities similar to human heritable skeletal disorder cleidocranial dysplasia (CCD) abnormalities[8]. On the other hand, tissue-specific Runx2 over-expression in transgenic mice results in decreased bone density, bone fractures and osteopenia[7,9,10].

Bone remodeling

Bone remodeling, the continuous bone reconstruction is of major importance for conserving bone structural integrity as well as for the bone to perform its metabolic role by modulating calcium and phosphorus levels in the body[1].

Shortly, bone remodeling activation depends mostly on local factors and their effects on mesenchymal progenitor cells. Bone reconstruction initiates with osteoclasts performing bone resorption and forming cavities inside the bone. At the end of this phase, osteoclasts produce the appropriate signals for the initiation of bone synthesis[1]. Osteoblasts quickly cover the cavity surfaces and synthesize new bone. Those two bone remodeling phases, bone formation and resorption are closely correlated and interconnected. This means that under normal conditions, the newly formed and the reabsorbed bone quantities are equal[11]. Impaired bone remodeling may lead in pathophysiological bone conditions like osteoporosis, Paget's disease, orthopedic disorders and osteopetrosis among others[1].

Research has shown that the GH-IGF-1 axis may also be of significance in the modulation of bone mass quantity and quality. More specifically, growth hormone (GH) is suggested to potentially play a role on bone remodeling[12]. However, the exact mechanisms through which GH acts on osteoblast biology have not been elucidated[12].

Role of RANK/RANKL/OPG pathway in bone remodeling

The receptor activator for nuclear factor κb (RANK)/ receptor activator for nuclear factor κb ligand (RANKL)/ osteoprotegerin (OPG) system comprises the main modulator of bone remodeling[13]. More specifically, pre-osteoclasts express RANK in their surface. Its ligand, RANKL, is produced in osteoblasts, stromal cells as well as activated T cells[14]. In osteoblasts and under steady-state conditions, vitamin D, parathyroid hormone and prostaglandins lead in induced RANKL expression. The binding of RANK and RANKL leads in osteoclast differentiation[15,16]. More specifically, during normal bone remodeling, RANKL is produced by cells of the bone marrow- supporting tissue and osteoblasts. RANKL binds to RANK on pre- osteoclasts resulting in their maturation and activation. Nuclear-factor κB (NF-κB), which is of importance in inflammation response, also plays a central role in osteoclast activation. NF-κB performs both aforementioned functions through regulation from interleukin-6 (IL-6). Pro-inflammatory cytokines play an important role in bone remodeling as indicated by the presence of interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) receptors on pre-and mature osteoclasts[17]. OPG is produced by osteoblasts and has the ability to bind to RANKL and block its functions resulting in decreased bone resorption[17,18].

MECHANOTRANSDUCTION

Bone remodeling and mechanotransduction

Bone remodeling is a strictly regulated process, largely modulated by the application of different mechanical stimuli or by metabolic stress on the bone[3].

More specifically, local mechanical stress leads in bone resorption as an initial response[19]. The nature of the mechanical stimulus is of importance in the regulation of bone remodeling since different types of mechanical stimuli result in different responses. For example, con-

stant repetitive application of mechanical force inducing high stress levels or unusual load distribution result s in elevated bone synthesis and high bone mass. Furthermore, short pauses between long periods of mechanical loading have been shown to enhance bone strength and structure[20]. However, static load, slow rates of pressure rotation as well as "predictable" pressure application, lead in decreased bone synthesis, enhanced bone resorption and thus low bone mass[21,22].

Bone remodeling and mechanostimulation have been shown to roughly follow these rules: Bone synthesis is promoted by dynamic and not static loading application. Short-term load applications are sufficient for adaptive response initiation and lead in increased bone formation whereas long-term load applications result in decreased bone synthesis and enhanced resorption[23,24]. In addition, the repentance of the same mechanical stimulus results in decreased response due to signaling prediction[25]. The application of these rules is evident in the effects of space microgravity, osteoporosis or paralysis on bone tissues, where bone loss is observed[20,26], as well as in the effects of tennis at a professional level on bone tissues, where bone growth is observed[27].

Mechanisms in mechanotransduction

Signals of mechanical nature induce in osteoblasts and osteocytes the production and secretion of different types of molecules, which modulate osteoblast differentiation and proliferation[3]. Such mechanical stimuli can include flow of fluids, strain of the substrate, membrane deformation or stimulation of integrins, vibration, altered gravity forces and compressive loading[3]. Bone remodeling functions, after the application of different mechanical stimuli, are locally regulated by cytokines and growth factors among other molecules. More specifically, IL-1β, TNF-α, prostaglandin E2 (PGE2)[26,28], IL-6, IL-8, RANKL, OPG[27,29-31], insulin-like growth factor (IGF), transforming growth factor β-1 (TGFβ-1) and fibroblast growth factor (FGF)[32,33] have been demonstrated to be induced after application of mechanical stimuli. Additionally, it has been shown that mechanical stimulation in osteoblasts results in increased mRNA levels of osteopontin, osteocalcin, platelet derived growth factor and collagen types I and Ⅲ[34,35].

Although some of the molecules taking part in mechanotransduction are known, the mechanisms behind it have not been fully elucidated.

The stage of osteoblast differentiation is shown to be of importance in osteoblast proliferation, apoptosis and translation of mechanical cues[36]. Furthermore, it has been shown that undifferentiated mesenchymal stem cells seem to respond more successfully to load application than mesenchymal stem cells that have already started to differentiate[37].

A diversity of molecules have been considered to play the role of mechano-sensors in differentiated osteoblasts: mechanical stimulation has been shown to lead in enhanced sensitivity and elevated open cation channels number[38,39], increased communication through gap junctions between osteoblasts as well as increased integrin production in osteoblasts[39]. Actin cytoskeleton abnormalities have been shown to prevent mechanical signaling and therefore the integrin network has been considered as the main candidate for transduction of mechanical signals[39]. On the other hand, a considerable number of research groups argue that cytoskeletal components involved in mechanotransduction differ depending on different types of stress or the response under study[39].

Integrins comprise transmembrane receptors connecting the extracellular matrix to the cytoskeleton[40]. Under mechanical signal application, integrins form complexes with molecules of the cytoskeleton with the help of the Rho family of Ras-related GTPases[40]. Rho family members also induce multiple kinase cascades and particularly mitogen-activated protein kinase (MAPK) cascades[40]. Rho and other Ras-related GTPases have been shown to play a role in osteoblast response after application of mechanical pressure[41]. More specifically, it has been shown that the continuous application of mechanical forces leads in deregulation of Rab and Rho GTPases activity in osteoblast-like cells[41].

Recently, another molecule, Polycystin-1 (PC1), was suggested to provide a link between environmental mechanical signals and their transformation towards biochemical signals. It has been shown that PC1 not only functions as a mechanosensor but that also conveys mechanical signals through the calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathway and thereby regulates osteoblast- specific gene transcription as well as osteoblast differentiation[42].

The primary cilium, a cellular sensory system, has also been demonstrated to be of importance in the transfer of mechanical signals as well as in mesenchymal stem cell differentiation. Additionally it was shown that the cilium modulates fluid flow mechanotransduction in human mesenchymal stem cells by maintaining fluid flow-induced osteogenic gene expression elevation and preventing fluid flow-induced increased proliferation[43].

Following the reception of mechanical cues, the signal conveying the mechanical conditions of the extracellular environment is carried towards the nucleus through MAPK kinases and more importantly through extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs)[44,45]. ERKs, which in human osteoblasts seem to be induced by growth factors, estrogen and fluoride among others[45], have been shown to play a significant role in osteoblast maturation and in osteoblast biology in general[45-49]. Furthermore, duration and strength of JNK/ERK signaling is indicated to be significant in gene expression[50].

Following ERK/JNK activation, the signal is transmitted to transcription factors that alter gene expression, like Jun and Fos family members[51]. In their turn, c-Jun and Fos family members interact to form activator protein-1 (AP-1) transcription factor, which has been shown to be of major importance in osteoblast differentiation[52] since it regulates the expression of collagen type I , osteocalcin, osteopontin and osteonectin[52].

Application of continuous mechanical pressure in osteoblast-like cells as well as osteoblasts resulted in increased production of AP-1 components through activation of MAPK cascades[41,53,54]. However, data on c-Jun expression after mechanical stimulation are inconclusive with some research groups arguing that human osteoblast-like cells after mechanical loading over-express c-Jun[53] whereas others have opposing results[55,56]. However, the above mentioned differences could be attributed to application of different stress type or usage of different cell system. Finally, different types of mechanical pressure applied on osteoblasts seem to result in different composition AP-1 and therefore regulate gene transcription accordingly depending on the extracellular signal applied[57].

Application of short-term mechanical pressure activates both JNK2 and ERK2, with following activation of downstream molecules, like c-Jun, which alter the expression of osteoblastic genes[54]. More specifically, it has been demonstrated that short-term continuous mechanical stimuli of physiological intensity in osteoblast-like cells activates JNK and ERK members resulting in enhanced AP-1 DNA binding activity on the human *L/B/K ALP* gene and thus osteoblast differentiation[54]. This is further evidenced by the observation that osteoblast-like cells receiving mechanical stimuli synthesized increased quantities of type 1 collagen and osteocalcin, markers of early osteoblast differentiation[58].

PGE2 production has been shown to be induced in osteoblast-like cells after mechanical stimulation[59] and in osteoblasts under the effect of physiological stress, growth factors, hormones, trauma or inflammatory cytokines and its production leads in cAMP-dependent IGF-1 induction in osteoblasts[3]. IGF-1 and IGF-2, in turn, induce osterix (Osx) transcription factor expression in osteoblasts[60], induce osteoblast function *in vitro* as well as lead in increased bone mass *in vivo*[61]. PGE2 is also shown to lead in increased Runx2 expression *in vivo*[62]. Downstream of PGE2, TGF-β expression, which leads in proliferation of osteoblasts and extracellular matrix synthesis[63], has been found increased in human osteoblast-like cells under mechanical stimulation. Furthermore, TGF-β receptor 1 comprises a Runx2 target in osteoblasts[64]. Those two observations combined explain why Runx2 knockout mice demonstrate characteristic abnormal extracellular matrix formation due to decreased number of mature osteoblasts[65,66].

Nitric oxide (NO) production in osteoblasts is another response to mechanical stimulation. NO functions through the MEK/ERK cascade by binding to a regulatory site on Ras leading in cell proliferation and extracellular matrix production[67]. Following, cyclooxygenase 1 (Cox1), Cox2, ERK1 and ERK2 are activated and result in bone matrix formation[68].

Additionally, signals of mechanical nature have been shown to promote vascular endothelial growth factor-, bone morphogenetic protein 2 (BMP-2)- and BMP-4- dependent and PGE2- independent increased expression of IGF-1[69]. BMPs result in bone synthesis in osteoblasts[70] and BMP-2 expression promotes Runx2, Osx and Dlx5 expression[71].

Mechanical cues also promote the expression of genes that encode for c-Fos, early growth response factor 1 (Egr-1) and basic fibroblast growth factor (bFGF) which have been shown to promote cell growth in MC3T3-E1 osteoblasts[22].

The nature the mechanical signal determines whether bone or cartilage formation will occur[72]. More specifically, application of pressure of high frequency and low intensity in bone cells *in vitro*, results in elevated extracellular matrix (ECM) disposition and thus increased bone formation[73]. On the contrary, mechanical loading of high intensity on osteoblasts leads in BMP extracellular antagonists expression and therefore results in inhibition of osteoblast development[74]. In addition, the application of continuous mechanical forces on osteoblastic cells *in vitro* promotes inflammatory cytokines and their receptors expression[75]. More specifically, IL-1b production is found elevated under such mechanical stimuli, and is accompanied by RANK-RANKL signaling pathway activation and thus bone resorption[76]. Stimuli from short periods of fluid flow or cyclic substrate tension at physiological intensity levels promote osteoblast proliferation and survival[77]. Mechanical signals of physiological intensity levels are associated with survival of human osteoblasts and several studies suggest that pro-survival proteins promote the production of survival factors like IGF-1 or IGF-2 and activate estrogen receptor[78]. It has also been shown that gravitational force maintains osteoblast survival whereas when gravitational force is not taking place, osteoblasts are led to apoptosis through reduced DNA binding of an important for survival transcriptional factor[18]. *In vivo*, the absence of mechanical signals promotes osteoblast apoptosis and thus osteoporosis[72]. The application of excessive mechanical force *in vitro* leads in cell detachment from their adhering surface[79] as well as in a form of programmed cell death called anoikis[80].

Mechanical stimulation in osteocytes has also been under investigation since it may lead in better mechanotransduction understanding and may represent a potent therapeutic target against bone degenerative diseases. Recent studies have underlined the role of osteocytes in bone remodeling since their absence in mice led in fragile bones, microfractures, deregulated osteoblast functions, bone loss in the trabeculae as well as adipose tissue proliferation in the marrow indicating an aging skeleton. In addition, these mice could not experience bone loss due to unloading, an event that indicates osteocytes' importance in the procedure of mechanotransduction[81] (Figure 1).

Runx2 in mechanotransduction

Runx2 which is known to play a significant role in osteoblast differentiation has been shown to be the recipient of mechanical signals in human osteoblast-like cells[82]. As it has been demonstrated, continuous mechanical stimuli of low intensity in human osteoblast-like cells of the periodontal ligament (PDL) result in elevated Runx2

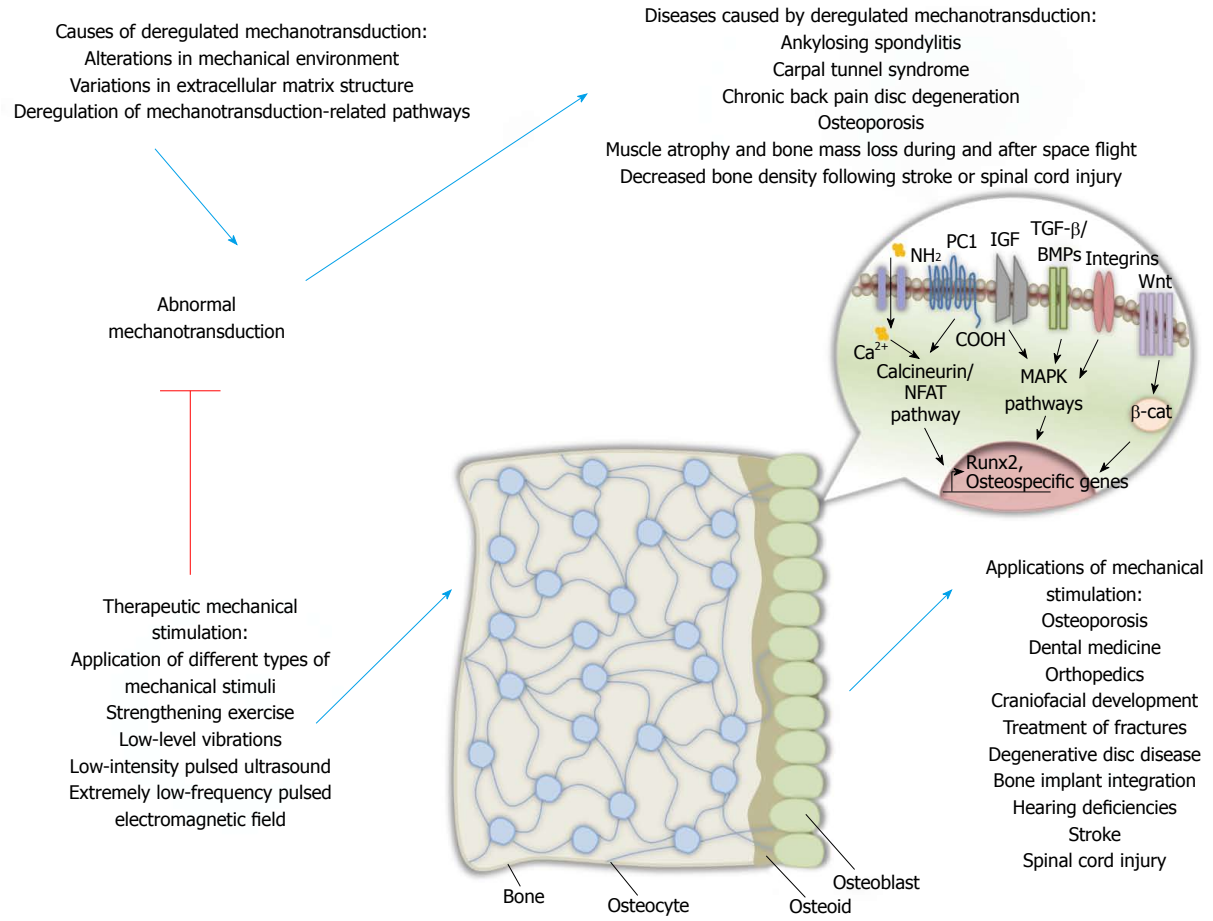

Figure 1 Mechanotransduction: Deregulation, associated disorders and therapeutic implications. Causes and effects of distorted mechanotransduction and the role of mechanical stimulation in the treatment of various pathophysiologies. PC1: Polycystin-1; IGF: Insulin-like growth factor; TGF-β: Transforming growth factor β; BMP: Bone morphogenetic protein. NFAT: Nuclear factor of activated T-cells; MAPK: Mitogen-activated protein kinase; Runx2: Runt-related transcription factor 2.

expression and DNA- binding capacity. The mechanical signal, according to the researchers, initiates at the plasma membrane and more specifically from integrins and travels towards the nucleus through MAPK cascades. In the nucleus, the signal targets Runx2 and induces its expression[82]. More specifically, Runx2 demonstrates increased expression at both mRNA and protein levels as well as elevated DNA binding activity. During this process, ERK1 and ERK2 are activated in a parallel manner with the Runx2 DNA- binding capacity elevation. After their activation, ERKs interact, phosphorylate and activate Runx2 *in vivo* causing osteoblast maturation[7,82].

Runx2 expression depends on an autoregulatory mechanism[83]. More specifically, activated by mechanical stimuli ERKs phosphorylate and activate already existing Runx2 molecules. Those activated Runx2 molecules bind to Runx2 promoter inducing Runx2 expression[82]. In addition, a canonical AP-1 binding site has been found in Runx2 promoter which potentially plays a role in the regulation of Runx2 expression. AP-1 and Runx2 proteins have also been shown to interact and regulate collagenase-3 expression[84].

NF-κB transcription factor in mechanotransduction

NF-κB transcription factor which is implicated in inflammatory response signaling[31] also plays a crucial role in osteoclast formation and thus bone resorption[85]. NF-κB, which is activated either through the RANK-RANKL system or potentially through integrins that transmit signals of mechanical nature to src-kinases[86], besides its role in osteoclast maturation, may be implicated in osteoblast differentiation under mechanical stimulation. This is indicated by the fact that NF-κB is found to be activated and then translocated in the nucleus of osteoblasts that receive mechanical stimuli[26,87] where it has been hypothesized to promote the transcription of osteoblast-specific genes.

MODEL SYSTEMS IN MECHANOTRANSDUCTION STUDY

The *in vitro* study of mechanostimulation in osteoblasts, has been made possible with the usage of osteoblast-like cells that are acquired either from healthy tissue (human PDL or mouse MC3T3-E1 calvaria cells) or from osteosarcomas (MG-63, SaOs cells). Different types of mechanical stimulation are applied on the aforementioned cell models, each causing a different response in osteoblast-like cells[3]. Such types of mechanical stimulation include fluid flow, four-point bending and substrate

stretch, gravity force, vibration, magnetic bead twisting and atomic force or shockwaves among others[88].

Periodontal ligament (PDL) cell system is a helpful model for the study of mechanotransduction signaling cascades in osteoblasts[89]. More specifically, PDL cells are undifferentiated mesenchymal fibroblasts[90] that bear all the characterized properties of osteoblasts. Furthermore, these cells are adapted to receive mechanical pressure, either because of physiological conditions or orthodontic treatments. Under specific conditions, PDL cells have the ability to differentiate towards more specialized cells capable of taking part in the regeneration and repair of the periodontal ligament as well as its surrounding hard tissue[91].

Furthermore, three dimensional (3-D) constructs, like polydimethylsiloxane microdevices and human trabecular 3-D bone scaffolds, have been used to investigate the effects of mechanical stimulation on osteoblasts[92].

Scientists are trying to develop an effective way to monitor the levels and characteristics of mechanical pressure applied as well as a way to measure the rates of tissue regeneration. In order to achieve the first part, scientists have made either fixation devices with different mechanical pressure characteristics and then monitor their effects *in vivo* or custom-made devices that accurately control the mechanical stimulation characteristics. With the first type of devices they are able to study bone tissue regeneration under more physiological conditions while with the second they assess the effects to a specific loading signal[93]. In order to study the effect of mechanical signals on healing processes at organs, it is necessary to develop techniques to assess their mechanical environment *in vivo*. Today, we have found ways to determine loading applied on the affected limb[94], load distribution between implant and bone[95-97], and assess interfragmentary movements[94,98] but the development of techniques to study the intermediate steps and not only the final outcome of loading are imperative.

MECHANOTRANSDUCTION IN BONE DISEASE

As mentioned before, deregulated bone remodeling is the main cause of a number of bone diseases. Bone remodeling abnormalities may be due to genetic alterations. For example, a mutant *runx2* gene can result in human heritable skeletal disorder CCD[99,100]. A mutation in *runx2* gene may also lead in cancer metastasis to bone tissues since Runx2 is responsible for the expression of genes that are implicated in cancer development and more specifically, in cell metastasis in bone. Among those genes regulated by Runx2 are those encoding matrix metalloproteinases (MMPs) MMP-9 and MMP-13 as well as osteopontin and bone sialoprotein[101]. Abnormal mechanotransduction due to lack of mechanical loading or other causes may result in bone remodeling deregulations like ankylosing spondylitis, carpal tunnel syndrome, chronic back pain disc degeneration and osteoporosis.

Recent studies have shown that annulus fibrosus (AF) cells that originate from non degenerative tissue respond to cyclic tensile strain through IL-1 and IL-4 dependent mechanisms, something that does not apply in AF cells coming from degenerative tissue[102]. Furthermore, annulus fibrosus cells from degenerative discs have been found to have little capacity to successfully respond to application of mechanical stimuli and exhibit an intense response to inflammatory stimuli. The above observations may explain the different responses observed in patients with intervertebral disc degeneration after specific therapies[103].

During space flight, astronauts are exposed to microgravity and thus altered mechanical stimuli are applied on their skeletons. As a result, their muscles atrophy and their bones experience bone mass loss. Short exposure to microgravity has been shown to result in increased bone resorption evidenced by the urinary calcium excretion observed[104]. Under long periods of microgravity, the structural alterations occurring in bones have even more crucial effects on bone strength than was previously thought while counteracting measurements like exercise seem to have little or no effects[104]. The mechanism behind bone loss is not yet clarified but probably is a result of decreased hydrostatic pressures and thus decreased intramedullary pressure which may lead in reduced fluid flow shear stresses on osteocytes and thus enhanced bone loss. Since exercise does not seem to prevent bone loss, it has been suggested that the decreased hydrostatic pressure may result in impaired mechanosensitivity in the bone tissue. Furthermore, other physiologic alterations on the body under reduced gravity conditions may contribute to the observed bone loss in co-operation with the reduced hydrostatic pressures like low vitamin D levels, oxidative stress, radiation exposure and acidosis[105-109].

Neurologic injury results in bone loss in the affected paretic limb whereas the other limb is characterized either by reduced or increased bone mass. Those effects are probably due to alterations in muscle mass and strength and load pressure applied. More specifically, strokes result in decreased bone density mostly in the paretic limb and its effects are more intense in the upper extremities. The pattern of bone loss observed in stroke patients is generally limited to the paretic side and is more evident in the upper extremities than in the lower extremities. The pathogenesis of the observed bone loss after stroke probably depends between others on immobilization, duration of paresis, loss of muscle activity, endocrine disorders, nutritional deficiencies as well as medications[110].

Following spinal cord injury, bone loss is observed in pelvis and lower extremities of paraplegics and in the upper and lower extremities of tetraplegics after spinal cord injury[111]. Those effects are predominantly observed in trabecular bone. Recent data indicate the presence of endocortical resorption without periosteal synthesis[112]. Absence of mechanical stimulation, muscle contraction, neuroendocrine alterations as well as neural innervation alteration are probably responsible for the observed bone

loss after those types of injuries[113,114] (Figure 1).

MECHANOSTIMULATION IN THERAPY OF BONE DISEASE

Pharmaceutical treatments like anabolic treatments or treatments with anti-resorptive agents have been the norm in order to achieve increased bone density until now[3]. Nowadays, mechanical stimulation is considered to be of great importance in designing new therapies for bone diseases, avoiding this way the unwanted side effects of pharmaceutical products.

A number of studies demonstrate the role of mechanostimulation in acquiring a higher bone mass quantity and thus its role in treatment of bone diseases. For example, it has been shown that low intensity mechanical signals result in bone remodeling activation and increased bone mass and that following a period of time confer regenerative abilities to bone tissues[115]. It has also been observed that mechanical signal application on PDL and osteoblast cell lines leads in enhanced OPG expression[116,117] and therefore in RANK-RANKL signaling interruption which results in decreased osteoclastogenesis. Furthermore, mechanical stimulation has been shown to activate Cox enzymes and prostaglandins which reduce RANKL production and thus block bone resorption *in vitro*[77,118]. Mechanical stimuli have also been demonstrated to activate the Wnt-b-catenin pathway on osteoblasts resulting in enhanced osteoblast differentiation and bone synthesis[119]. Studies on three dimensional models have showed that osteoblasts receiving dynamic application of mechanical pressure, expressed elevated ALP, Runx2 and osteocalcin levels[120,121]. Additionally, application of mechanical pressure resulted in increased mineralized matrix production in 3-D, partially demineralized bone scaffold-cultured human bone marrow stromal cells[122].

Considering the aforementioned and other results, researchers have turned to mechanical stimulation in order to design treatments against bone diseases which will avoid the undesirable effects of pharmacological treatments[115]. Application of mechanostimulation has already a variety of applications in dentistry, orthopedics, the craniofacial development and treatment of fractures.

More specifically, strengthening exercises in osteoporotic patients has been shown to result in increased bone mineral content[123] and physical exercise has been observed to prevent post-menopausal and age-related ECM bone mineral decrease[124]. Moreover, other types of mechanical stimulation like low-level vibrations at intensity safe for the bone integrity may play a protective role in osteoporosis[125]. A functional mechanical environment seems to be of importance in the treatment of degenerative disc disease as well as other skeletal deregulations[126]. Mechanical signals of specific ratio[127], form[128] and intensity in osteoblasts have also been shown to be beneficial in bone fracture treatment[128]. Additionally, low-intensity pulsed ultrasound has been indicated to promote osteoblast differentiation and bone formation in bone fractures[129]. Extremely low-frequency pulsed electromagnetic field has been demonstrated to result in osteoblast proliferation and maturation[130].

In addition, mechanostimulation was found to have positive effects in bone implant integration by modulating osteoblast differentiation through regulation of Cbfα1 as well as osteocalcin levels. Cbfα1 and osteocalcin levels were shown to be frequency-, magnitude-, and duration of mechanical application- dependent. Furthermore, osteoblast cells under strain in the implant seem to produce factors that have the ability to activate DNA synthesis and thus cell proliferation in a larger scale than non-strained cells[131].

Mechanical stimulation has also its applications in the treatment of hearing problems. For example, SPAHA, which comprises a novel bone conduction hearing device, whose effects are accomplished through elastic bending of the bone and not the application of a point force which results in cochlea vibration as previous devices used to do[132].

Exercise has not been shown to meliorate bone loss in space flights until now[104]. Furthermore, there is no indication that osteoporosis drug therapies would be successful during or following space flight. Exercise seems to be helpful in increasing bone density after stroke or spinal cord injury according to a recent study[133,134]. Bisphosphonates have been shown to be able to prevent bone loss after a stroke[134]. Mechanical stimulation may have some positive effects on preventing bone loss after spinal cord injury, with early application demonstrated to bear better results[135,136]. Furthermore, bisphosphonate early administration after spinal cord injury may be able to prevent bone loss[137].

Researchers have investigated whether sympathetic nervous system inhibition could be beneficial against bone loss in osteopenia induced by absence of mechanical signals. They found that its inhibition led in blockade of neurectomy-induced bone resorption but further studies need to be conducted[138].

Although mechanical loading is thought to be an anabolic beneficial procedure against osteoporosis, abnormal mechanotransduction in conjunction with age seem to counteract its beneficial effects in elderly people. Recently, a research group presented an agent-based model of real-time Ca^{2+}/NFAT signaling in bone cells that successfully described periosteal bone synthesis induced by different types of mechanical stimulation in young and aged animals. The model demonstrated age-related pathway changes being responsible for the decrease in bone synthesis during senescence. This way the group managed to identify important pathway alterations that comprise potent therapeutic targets. In accordance, the researchers applied an *in vivo* intervention and showed that application of mechanical stimuli along with Cyclosporin A can prohibit the decrease in bone synthesis in the bones of elderly people. This study not only provided a potent inexpensive treatment for osteoporosis in the elderly but also demonstrated the significance of real-time cellular

signaling and *in silico* techniques in studying, intervening and treating bone diseases like osteoporosis[139].

The primary cilium was shown to modulate fluid flow mechanotransduction in human mesenchymal stem cells by maintaining fluid flow-induced osteogenic gene expression elevation and preventing fluid flow-induced increased proliferation[43]. Therefore, fluid flow systems may be effective in designing techniques to develop bone-like tissues for bone regenerative purposes. Furthermore, the role of cilium in developing techniques that imitate loading in order to treat bone loss in bone diseases needs to be investigated. Last but not least, studying the events taking place during acute proliferation of mesenchymal stem cells with not functional cilia receiving mechanical cues could help in understanding the mechanisms behind ciliopathies and cystic diseases[43] (Figure 1).

CONCLUSION

Bone remodeling is of major importance for the proper structure and metabolic functions of the bone. Deregulations in bone remodeling can result in a variety of bone diseases like osteoporosis, hyperparathyroidism, hyperthyroidism, Paget's and osteopetrosis among others. Therefore, the investigation of mechanisms and pathways behind bone remodeling and mechanotransduction, which comprises of the most important variables of bone remodeling, is of great significance.

There is a lot that we don't know about bone biology and bone diseases as well as the implication of mechanical signals in the aforementioned procedures. The better understanding of the underlying mechanisms will potentially result in designing a successful strategy for treating bone diseases, avoiding the unpleasant side effects of conventional treatments like the administration of pharmaceutical substances. Furthermore, it will help us design techniques to successfully predict and prevent bone diseases when possible.

Undeniable is the necessity of innovative new ways to monitor bone density, to identify hormonal or metabolic risk factors for bone loss, to develop effective ways to apply mechanical stimulation with successful results against reduced bone density, to assess the effect of newly developed anabolic drugs against osteoporosis and their effects on bone loss characterizing bone diseases due to absence of mechanical stimuli, as well as to develop trials investigating the improvement of bone health under the afore mentioned conditions. In addition, the study on the effects of mechanostimulation on bone tissue and organ healing is of great significance for future interventions. In order for this to be achieved, we need to develop an effective way to monitor the levels and characteristics of mechanical pressure applied on bone tissue, a way to measure the rates of tissue regeneration as well as techniques to assess mechanical environment of organs *in vivo*[106].

Currently, researchers have started using mechanostimulation with encouraging results for certain bone conditions but further study is required. Mechanostimulation is considered to comprise the future in treating bone diseases that have their origin in absence of mechanical cues. Further investigation of the molecular players and pathways involved in mechanotransduction and bone remodeling will amplify our knowledge and understanding of these processes and help us build successful prevention, prediction and treatment strategies for a variety of bone diseases.

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REVIEW

Role of oral-fluid based measles diagnostic methods for measles global elimination

Wondatir Nigatu, Antoine Nsabimana

Wondatir Nigatu, Ethiopian Health and Nutrition, Research Institute, Addis Ababa 1142, Ethiopia
Wondatir Nigatu, Applied Biology Department, Kigali Institute of Science and Technology, BP Kigali 3900, Rwanda
Antoine Nsabimana, Faculty of Science, Kigali Institute of Science and Technology, BP Kigali 3900, Rwanda
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Correspondence to: Wondatir Nigatu, Professor, Applied Biology Department, Kigali Institute of Science and Technology, Avenue de l'Arměe, BP Kigali 3900, Rwanda. wnigatu1891@yahoo.co.uk
Telephone: +250-783-628866 Fax: +250-252-571925
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Abstract

Measles eradication is biologically feasible. There is an availability of a safe, effective and inexpensive vaccine; a proven elimination strategy; high Local demand; and an effective global partnership and initiative to support vaccination. Measles eradication is a cost-effective scenario and a good investment to avoid expensive epidemics and save those children die due to measles. Laboratory investigations are indispensable to monitor the progress of measles elimination. This role will require the development of more sensitive diagnostic methods suitable for diagnosis and surveillance, genetic analysis of measles strains and a technology which is transferable worldwide. Measles diagnosis relies increasingly on serological tests. The practical utility of oral-fluid methods (antibody and genetic) in evaluating and refining measles immunization programs would, additionally, provide support for a global surveillance initiative. The utility of in a population survey, in a vaccine sero-conversion study and application in molecular epidemiological use is demonstrated in this review. It is to be hoped that this review will assist in the wider uptake and acceptance of methodology in both developed and developing country situation. More research needed for further evaluation of a recently developed point-of-care test for measles diagnosis: detection of measles-specific IgM antibodies and viral nucleic acid for wider use oral-fluid methodology. There is a strong case and imperative for the promotion of methods by World Health Organization in its global program of control/eradication of measles over the coming decade.

Key words: Measles elimination; Oral-fluid test methods; Laboratory diagnosis

Core tip: Laboratory investigations play a critical role in monitoring the success of measles elimination strategies. The role requires the development of more sensitive diagnostic which is transferable worldwide. Measles diagnosis relies increasingly on serological tests. Promotion of the use of oral fluid as viral diagnostic alternative to serum may be of advantage in communities where reliable age-specific notification and vaccination data are unavailable or in groups that are "hard to reach". This review will assist in the wider uptake and acceptance of oral-fluid methodology in both developed and developing country situation for global measles elimination.

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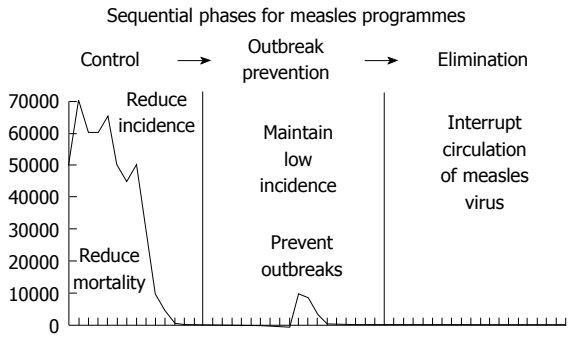

Figure 1 Phases for measles control/eradication programmes.

PHASES OF MEASLES CONTROL AND ELIMINATION

Measles is a highly contagious disease caused by a virus. It is one of the leading causes of death among young children. In 1980, before widespread vaccination, measles caused an estimated 2.6 million deaths each year. It remains one of the leading causes of death globally, despite the availability of a safe and effective vaccine. It is estimated that approximately 158000 people died from measles in 2011, mostly children under the age of five[1].

Based on implementation of a combination of vaccination and surveillance strategies, countries are considered to be in 1 of 3 stages: control, outbreak prevention, or elimination[2,3] (Figure 1).

MEASLES CONTROL

Control is defined as the reduction of disease incidence and/or prevalence to an acceptable level as a result of deliberate efforts, requiring continued interruption measures. In the control stage, the objective is to achieve high routine coverage with 1 dose of measles vaccine among infants to reduce measles morbidity and mortality. To accelerate measles control in large urban and other high-risk areas with a substantial proportion of unvaccinated children and measles associated deaths, mass vaccination campaigns targeting children aged 9 mo to 3-14 years have been recommended[4,5]. Countries in all regions have committed to the mortality reduction goal. Global measles deaths are decreased by 78% between 2000 and 2008, averting an estimated 4.3 million deaths[6,7]. The Southeast Asia region is already exceeding 90% measles mortality reduction[8].

MEASLES OUTBREAK PREVENTION

Measles outbreak prevention aims to maintain low incidence and prevent outbreaks by the administration of supplemental doses of measles vaccine through mass vaccination campaigns. As programmes plan for elimination of measles, a high coverage of single dose vaccine with supplementary immunization is assumed be sufficient to interrupt transmission[9]. A second dose is required to eliminate susceptibles from the population and interrupt measles transmission[10]. The Africa region is adopting to raise routine vaccine coverage to at least 80% and using supplemental campaigns in all non-polio-reservoir countries by 2003[11]. The Western Pacific Region from 1996 to 2009, 235 million persons received measles vaccine during 94 immunization campaigns in 30 countries and areas[12]. In the same region during 2009, 32 countries and areas provided 2 routine doses of measles vaccine[12]. The steady increase in routine measles coverage is shown from 71% to 82% globally between 2000 and 2009[8]. Between 2000 and 2008, administration of more than 600 million doses of measles vaccine in mass vaccination campaigns were made globally[7,13].

MEASLES ELIMINATION

Elimination is defined as the reduction of endemic incidence of a disease to zero as a result of deliberate efforts, requiring continued control measures. An alternative approach to documenting measles elimination are molecular evidence to confirm the lack of a circulating endemic genotype for at least one year and maintenance of 95% coverage of one dose of measles-containing vaccine, with an opportunity for a second dose[14]. It is well understood that laboratory testing and confirmation of suspected measles infection is crucial in countries that are in elimination phase of measles[15-17]. Generally, all 6 World Health Organization (WHO) regions have committed to measles elimination, and 5 (except Southeast Asia) set target date to move from regional measles mortality reduction to the regional elimination of indigenous transmission[6].

GLOBAL PROGRESSES TOWARDS ELIMINATION

Although there has been tremendous success in the reduction of measles endemic incidence in many countries with measles elimination, the total interruption of measles transmission remains a major challenge due to importation of measles cases to America and Europe regions[18-20]. For example, the ongoing transmission of endemic measles was declared eliminated in the United States in 2000[21]. However, within five months period starting from January to May 2011, 118 cases were reported in the United States in which 46% of the cases were imported[18]. The elimination of measles deaths in Southern Africa in 2000 joins the region of the Americas to be free from measles deaths[22,23]. However, from July 2003 to November 2005, 1676 laboratory-confirmed measles cases were reported in South Africa[24] and silent causalities' the disease was also reported[25].

The WHO Europe strategic plan for measles 2010-15 sets targets of 90% measles vaccination coverage, and reductions in the number of cases to fewer than five per million and in mortality by 95% compared with 2000 levels[26,27]. The 37 countries and areas of the WHO Western Pacific Region have targeted measles for elimination by 2012[12].

Between 1997 and 2011, the goal of interrupting measles transmission was adopted toward the elimination of measles in the Eastern Mediterranean Region (EMR). For the 22 EMR member countries, routine coverage with the first dose of a measles-containing vaccine increased from 70% in 1997 to 82% in 2009. Reported measles cases decreased by 86% during 1998-2008, and estimated measles mortality decreased by 93% during 2000-2008, accounting for 17% of global measles mortality reduction during that period. Despite these successes, EMR was not being able to achieve measles elimination by the end of 2010[28].

Many Progresses have been achieved toward measles elimination in the People's Republic of China between 2000-2009 and in the Russian Federation between 2003-2009[29,30]. Globally, the number of measles deaths worldwide fell by 78% between 2000 and 2008, from an estimated 733000-164000[31]. Despite the efforts measles elimination, measles remains a disease still endemic in many parts of Europe[32]. For instance, between 2009 and 2011, Austria, France, Germany, Ireland, Italy, Greece, the Netherlands, Spain, Bulgaria, Norway and United Kingdom have all seen outbreaks[32-41].

Estimates indicate that almost a quarter of all lives saved annually towards achieving Millennium Development Goal 4 are the result of progress towards achieving a 90% reduction in measles deaths [7,10,13].

STRATEGIES FOR MEASLES CONTROL AND ELIMINATION

Key strategies for the local elimination/total eradication of measles as a disease are as follows. The spread of measles infection through a population requires that a chain of invectives should be maintained. Protection against this spread of infection can be taken at two points. First, the route from susceptible to recovered (return to immunized state after vaccine uptake) can be short-circuited by the establishment of immunization[42-45]. Second is to interrupt the mixing of invectives (carrier of the infections) and susceptible with protective barriers (*e.g.*, isolation)[46]. Incidence rises as susceptible individuals enter the population. Acquisition of immunity through exposure to the wild virus or vaccination decreases the number of susceptible individual in the population and measles incidence falls [47]. The greatest potential is with vaccination.

Acquired immunity after measles illness is permanent. Live attenuated measles virus, when administered at recommended ages, produces about 85% immunity after one dose and greater than 90% immunity after two doses[5,48,49]. Vaccine-induced immunity is long lasting and protective to all the diverse geographic origin strains. Widespread vaccination has resulted in interruption of measles virus transmission in a number of countries. For instance, the Gambia in 1968-1969, the English speaking Caribbean islands, Cuba, Chile, United States over short periods in 1993, 1995, and 1996[50,51]. Similar achievements were obtained in England and Wales through 1995-2000[20]. Estimate indicates increase in routine measles coverage from 71% to 82% globally between 2000 and 2009, and from 56% to 73% in the 47 countries with the greatest burden of measles deaths[7,8].

The success of recent mass vaccination campaigns in these countries has suggested that global eradication of measles is possible biologically, technically, and operationally[19,52]. Reaching this goal will require continued commitment to increase vaccination coverage levels with a co-coordinated global effort.

Vaccine investments rose from donors in United Kingdom, Japan, United States, *etc.,* to provide additional funding to the Global Alliance for Vaccines and Immunization (GAVI) for its childhood immunization program save many children's lives. Vaccination is one of the most cost-effective health interventions[53]. Studies show that measles eradication by 2020 was found to be the most cost-effective scenario globally[54].

Programmatic and technological innovation will be needed to sustain recent successes in reduction of the global burden of measles. Delivery of the measles vaccine through the respiratory tract could help this effort[55]. It has many advantages compared to the injectable vaccines in which the major one can be stated as follows[55-58]. Respiratory delivery generates robust local and systemic immune responses which resulted in superior and longer lasting protection and boosting better responses in seropositive people than are injectable vaccines[57,58]. This route is less likely to be blocked by maternal antibodies in infants than is a subcutaneous measles vaccine. Aerosol administration of vaccines needs fewer skills than injectable vaccines. Use of non-injectable vaccines reduces the likelihood of unsafe disposal and reuse of syringes in immunization program.

An important component of the measles control and elimination strategy is information obtained from laboratory. Currently the WHO Global Measles and Rubella Laboratory Network (LabNet) include 690 laboratories serving 183 countries[59].

MEASLES VACCINATION

Different factors affect the response to immunization, such as, age and maternal antibody level[60]. Wesley *et al*[61] reported that the response to measles immunization was delayed among malnourished children. The optimal age at delivery of measles vaccine depends upon the relationship between the average age at infection and the rate of loss (average duration) of maternal antibodies specific to measles[62,63]. Maternal antibodies typically provide protection during the first 6 mo of life, but often longer[63,64]. Interference with the replication of vaccine virus is frequently still seen at the age of 12 mo[65]. As a consequence vaccination in the first year of life gives inadequate immunity to measles, meaning the earlier at the age of vaccination the lower the sero conversion rate[63,64]. The requirement for delay until maternally derived antibodies vanish is an impediment for early vaccination.

The duration of maternally derived immunity in a child depends on the mother's antibody titer, the efficiency of transfer across the placenta and the rate of catabolism in the child[66,67]. A child exposed to many infections makes a large variety of immunoglobulin G (IgG); in order to keep the total blood IgG level in the normal range, catabolism is accelerated and passively acquired antibodies are swept out at an accelerated pace. In this way, early susceptibility to measles is strongly correlated with low economic status[67]. To meet this challenge age cross-sectional sero-epidemiological surveys and sero conversion studies are important for recommending the proper age for vaccination. An evaluation of the routine immunization program in Ethiopian children, reported here[68], gives support for the WHO recommended age for measles vaccination at 9 mo[69]. The ability of a measles vaccine to induce an immune response, particularly in the presence of maternal antibody, varies according to the strain and the dose of vaccine[63].

Acquired immunity after measles illness is permanent. Live attenuated measles virus, when administered at recommended ages, produces about 85% immunity after one dose and greater than 90% immunity after two doses[5,48,49]. Vaccine-induced immunity is long lasting and protective to all the diverse geographic origin strains.

Developed and developing countries of the world have different measles vaccination policy. In developed countries children are immunized at the age between 12-18 mo (depending up on the policy of different countries), as part of a three-part mumps and rubella (MMR)-vaccine. The vaccination is not given earlier than this because children younger than 12 mo usually retain anti-measles immunoglobulin's transmitted from the mother during pregnancy. A second dose is usually given to children between the ages of four and five. In developing countries where measles is highly endemic, it is recommend that two doses of vaccine be given at six months and at nine months of age. Serological studies in developing countries have shown sero-conversion rates following immunization at age 9 mo of 80%-90%[70,71]. Generally, the two-dose schedule is beneficial when there is a need to increase net vaccine efficacy, after coverage has been maximized with a one-dose schedule[64,72,73].

Role of laboratory for measles control and elimination

Laboratory investigation will play a critical role in monitoring the success of measles control strategies[15,16,59,74]. This role will require the development of more sensitive diagnostic methods suitable for diagnosis and surveillance, genetic analysis of measles strains and a technology which is transferable worldwide. Measles diagnosis relies increasingly on serological tests[75]. Serum based diagnosis can be made by virus isolation, by demonstration of a significant increase in specific IgG titers, or by the detection of anti-measles virus (MV) IgM antibodies by using radio-immunoassays (RIA), enzyme-linked immune sorbent assays (ELISAs) and direct or indirect fluorescence-antibody techniques[10,76,77]. Genetic characterization of wild-type measles viruses from different types of specimen sources provides a means to study the transmission pathways of the virus and is an essential component of laboratory-based surveillance[78-82].

The effectiveness of an immunization program can be evaluated though serological survey methods. Using dried blood spot (DBS) sample-drops of whole blood collected on filter paper from a simple finger prick-provides a minimally invasive method for collecting blood samples in nonclinical settings for serological and genetic analysis of measles[83-87]. The measles laboratory network required the use of alternative sampling techniques for surveillance[88]. The need for techniques that obviate the requirement for blood sampling promotes the application of oral-fluid based methods to the evaluation of the immunization programs. Oral-fluid based testing has an advantage of convenience, avoidance of inadvertent transmission of blood-borne pathogens, ease of use in pediatric and geriatric populations; as well as the potential for blood-free home and work place collection of patient samples.

Of the "ten elements of surveillance" summarized by WHO[89] at least in six of them visa a VIS, morbidity reporting, epidemic reporting, laboratory investigations, individual case investigations, epidemic field investigations and surveys, the laboratory has a role in providing serological results for measles surveillance. This indicates that the general advantages of measles surveillance data depend in large part on laboratory results[59,74]. The requirement of blood specimens for laboratory results can limit the yield of data for measles surveillance. In this respect we need another source of human biological material for measles surveillance, which is inexpensive and simple to collect, acceptable to donor and collector and provides accurate representation of serological status. Oral fluid has been explored as a source of human biological material for surveillance of viral diseases[90,91]. It has clear advantages over venipuncture in surveillance and epidemiology of viral diseases. In the United Kingdom, oral-fluid sampling and screening has been used for the surveillance of measles, MMR since 1994[20]. This has permitted the impact of MMR vaccination program to be monitored and evaluated in a way which may not have been possible through blood collection alone. Measles serological surveys could play a role in the evaluation of immunization programs[92,93]. Immuno-serological cross-sectional measles surveys have particular importance to determine immunization program strategy in relation to age groups, geographic areas, socio-economic groups and risk population groups. Follow-up serological measurements in measles immunized persons has importance to determine the proportion developing immune responses, quality and extent of response, duration of response and level of protection against measles infection. Periodic measles serological surveys have advantage to identify groups who are not receiving measles vaccines or who have inadequate responses. The importance of sero epidemiology for such purposes is paramount although the necessity for vein puncture reduces the ease

Table 1 Samples for laboratory diagnosis of measles virus infections

Virus disease	Samples for virus isolation for detection of antigen	Samples for serology	Remarks
Acute measles	Blood (leukocytes), throat secretions (saliva/oral-fluid), conjunctival secretions, urine; skin biopsies	Acute and convalescent serum	Period of infectivity; prodromal stage until 1-2 d after rash; antibody rises occur at appearance of rash; in tropical measles, possibly prolonged virus excretion also in stools
Measles pneumonia	Blood (leukocytes), throat secretions, conjunctival secretions, urine	Acute and convalescent serum	Frequently no rash; prolonged period of infectivity
Acute measles encephalitis	Brain specimen (biopsy or autopsy specimen), cells in CSF	Serum and CSF	In most cases, no infectious virus is detectable; occasional local production of antibodies in the CNS
SSPE	Brain specimen (biopsy or autopsy specimen), cells in CSF, lymph node biopsy (?)	Serum and CSF	Virus antigen detected in CSF cell; virus isolation requires propagation of explants cultures and cocultivation with susceptible cells; hyper-immune antibody response; local production of antibodies in the CNS

Modified from Norrby *et al*[137]. CSF: Central spinal fluid; CNS: Central nervous system.

and acceptability of this method. New methods that obviate the requirement for blood sampling could further encourage the application of measles serological surveys for the evaluation of measles immunization program. To achieve the aforementioned roles at better performance work was undertaken for measles vaccination program evaluation and surveillance based on oral-fluid collection and screening methods[94]. The purpose of this review is, therefore, to explore the development and evaluation of oral fluid as a diagnostic specimen for measles virus with particular reference to the developing country setting. The technologies developed[68,77,81] have increased the level of sensitivity and specificity where salivary examination for measles IgG and IgM is practical and convenient. Using polymerase chain reaction (PCR) technology we found oral-fluid from measles cases to be useful in the molecular characterization of measles virus. Success of the measles vaccination program can be assessed using oral fluid specimens as markers of sero-conversion.

ORAL-FLUID AS CLINICAL SPECIMENS

Laboratory investigation will play a critical role in monitoring the success of measles elimination strategies. As we shall see in this review the role will require the development of more sensitive diagnostic methods suitable for diagnosis and surveillance, genetic analysis of measles strains and a technology which is transferable worldwide.

Measles virus can be detected from various clinical samples by using serological methods, cell cultures techniques or molecular techniques. Samples that can be collected at different stages of the measles infection for virus isolation and serological tests are outlined in Table 1.

The concentration of antibody in saliva was found at much lower levels compared to plasma[95]. This has limited its use as diagnostic specimen for viral immunological assays. However, research demonstrated that salivary antibody has two sources, the parotid and crevicular crevice, with different concentration levels of immunoglobulin's[95]. The transudate that comes from the gingival crevice, whilst being lower in concentration, closely reflects the immunoglobulin class and specificities of antibody found in plasma[91,96]. The major reason for this is that the majority of the antibody present in the transudate comes from the small capillary bed beneath the margin that separates the teeth and gum. These properties of crevicular fluid lead investigators for measurements of virological markers of immune activation as an alternative to serum.

The other problem associated to the use of saliva as a viral diagnostic fluid is the need of immunological assays that have higher sensitivity. The development of antibody capture assays, ^{125}I labeled (RIA) or ELISA, that are able to generate higher signals by capturing a higher proportion of the total immunoglobulin (present in the oral fluid) specific for the antigen under test, enabled saliva to be used for successful immunological assays[97,98]. Presently the production of purified nucleoprotein through Baculovirus expression[99] increases the utility of saliva in diagnostic enzyme immunoassays.

The value of oral-fluid in screening for human immunodeficiency virus infection is now well established with the use of IgG captures radioimmunoassay[100,101]. The methodology has been applied to oral-fluid diagnosis of measles, mumps, rubella, Epstein-Barr virus and hepatitis A and B infection[77,100-104]. Veterinarians found it useful for detecting feline immunodeficiency virus[105], and feline leukemia virus[106]. Hepatitis C virus antibodies can be able detected from oral fluid[107]. Its potential application in bacteria was demonstrated with the measurement of specific IgA antibody to *Bordetella pertussis* antigens in saliva for diagnosis of whooping cough[108]. Other possibilities were seen in the diagnosis of cysticercoids by measuring specific salivary antibody to *Taenia solium* larvae[47]. Measuring of specific IgA antibodies to gliadin is used as a screening marker for coeliac disease[109,110]. Methods that can detect microbial antibodies in oral fluid such as *Helicobacter pylori* antibodies have been developed[111]. The potential to use oral fluid as Porcine Reproductive and Respiratory Syndrome virus in swine, cardiac diagnostics, oral cancer, systemic diseases, water-borne diseases, alcohol and drug testing specimen has been the subject of considerable scientific interest[112-118]. Generally oral-fluid as diagnostic fluid has the following advantages: (1) humanitarian-the patients are spared the discomfort of

repeated venipunctures; (2) clinical- with less stress, non-risk of anemia, infection or thrombosis; (3) for children-saliva sampling is the technique of choice; (4) economic-patients can collect themselves, thereby saving technicians' time, samples may also be mailed, eliminating travel time; and (5) eliminates the issue of protection of privacy and adulteration during sample collection; the ease and low cost of collection are major benefits in large-scale studies.

STUDIES PERFORMED ON MEASLES ORAL-FLUID BASED TEST METHODS

The works so far done can be specifically summarized as follows: (1) The development of a GACELISA for the detection of measles specific IgG in oral-fluid, with performance (sensitivity and specificity) that makes it suitable for replacement of serum assays, particularly for estimating population immunity[77]. By comparison with the serum measles IgG assay, the oral fluid GACELISA had a sensitivity of 97.4% (95%CI: 95.9-98.2) and a specificity of 90.0% (95%CI: 81.9-94.3), with no significant differences observed by age group. It is concluded that the overall performance of the GACELISA was satisfactory, showing close agreement to the serum ELISA, and has potential to serve as an easily transferable tool for large scale epidemiological studies as required for the World Health Organization's program for the global control of measles; (2) The development of a MACELISA for the detection of measles specific IgM in oral-fluid, suitable in performance to replace serum assays[68]; Screening of sera was undertaken using commercial indirect ELISA kits, and of oral fluids using an in-house IgM-capture ELISA. Pre-vaccination serology showed 1.4% IgM positive, 2.0% IgG positive, and 97.0% sero negative; Post-vaccination seroprevalence of IgM and IgG was 91.3% and 85.0%, respectively, and 92.9% overall. The seroconversion rate was 92.6% (95%CI 88.2-95.7); Based on oral fluid results, 87.3% (95%CI: 82.0-91.4) of children showed specific IgM antibody conversion. These results are in support of the recommended age for measles vaccination in Addis Ababa, and show the merit of oral-fluid IgM screening as a non-invasive alternative to blood for assessing vaccine immunogenicity; (3) Demonstration of the use of these assays in the estimation of measles antibody (immunity) prevalence in the vaccine-targeted population and in monitoring the outcome of a measles vaccination program (routine and campaign) in a developing country setting[68,96-98]; (4) Demonstrate the utility of oral fluid to study the molecular epidemiology of measles virus in both developed and developing country situations in a period of accelerated measles control[81,82,109]; (5) Oral fluid for the serological and molecular diagnosis of measles in a developed country setting[109,119]. These studies demonstrate the use of oral fluid samples for the detection of measles virus in the United Kingdom and the Belgian measles surveillance system and other studies in the framework of the WHO elimination program; (6) Technical refinements of sample collection and laboratory screening of oral fluid, and, importantly, comparisons with existing methods based on serum prior to wider adoption of non-invasive methods. This work includes the evaluation oral-fluid relative to serum and DBS for the detection of measles specific IgM in suspected measles cases in relation to assay type and sample timing post onset of rash. Works done to assess the performance (sensitivity and specificity) of a commercial IgG antibody capture method for oral fluid in relation to currently used assays for serum/blood spots is in preparation for publication (Dr. Nigatu W personal communication); (7) The studies emphasize the potential and suitability of oral-fluid to substitute serum in estimating and monitoring measles IgG antibodies, during community surveys[118-120]; (8) Applicability of oral fluid collected onto filter paper for detection and genetic characterization of measles virus strains[119,121]. The former study showed molecular nested RT-PCR using oral fluid was validated against the standard assay on nasopharyngeal secretions and gave a sensitivity of 100% and specificity of 100%. The latter study demonstrate that oral fluid dried onto filter paper can be used for the detection and characterization of MV strains. Using this approach, an MV-positive sample by reverse transcriptase PCR could be obtained from 67% of serologically confirmed acute measles cases; (9) Determination of measles immunization status using oral-fluid samples [122]. The presence of antibodies in oral fluid specimens correlated with that in serum with sensitivity and specificity: measles, 97% and 100%, respectively. This study assessed protective antibodies to measles by means of an oral fluid sample with good reliability; and (10) Evaluation of the performance of a newly developed point-of-care test (POCT) for the detection of measles-specific IgM antibodies in serum and oral fluid specimens and to assess if measles virus nucleic acid could be recovered from used POCT strips[123]. With oral fluids POCT showed sensitivity and specificity of 90.0% (63/70) and 96.2% (200/208), respectively. Both *H* and *N* genes were reliably detected in POCT strips and the *N* genes could be sequenced for genotyping. Measles virus genes could be recovered from POCT strips after storage for 5 wk at 20-25 ℃. The POCT has the sensitivity and specificity required of a field-based test for measles diagnosis. However, its role in global measles control programs requires further evaluation.

PRESENT AND FUTURE APPLICATIONS OF MEASLES ORAL-FLUID METHODS

Present applications

Community surveys of measles specific IgG/IgM are useful to guide the design of measles control programs. For example these help in (1) defining levels of immunity to measles pre- and post-vaccination efforts, *i.e.,* assessing the effectiveness of the vaccination program; (2) identifying age groups in which a significant susceptible proportion remain; and (3) assessing sero-conversion rates following vaccination. Analysis of the genetic characteristics

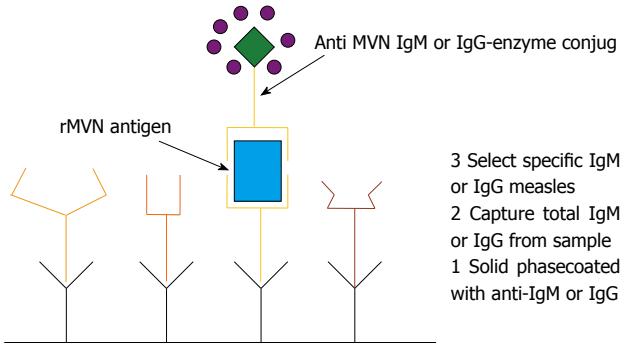

Figure 2 Principle of the"Microimmune" measles IgM or IgG Capture Enzyme Immuno-assay methodology. IgG: Immunoglobulin G; IgM: Immunoglobulin M; MVN: Medial vestibular nucleus.

of wild-type measles helps to elucidate the origin and transmission pathways of measles virus [124]. The genetic data when analyzed with other epidemiological data provides a means to assess the efficacy of measles control programs. For such molecular studies measles RNA can be detected by RT-PCR from isolates, oral fluid, blood, throat-swabs, urine collected from acute cases. There have been no systematic studies made to evaluate the relative sensitivity of these different samples. The primary role of this review is in the demonstration of the use of oral fluid as a clinical specimen for detecting IgG/IgM antibody for evaluating measles control strategies and the virus genome for molecular epidemiological studies.

Future technical development

The low concentration of IgG/IgM antibodies in oral fluid relative to other diagnostic specimens such as plasma[95] demanded the development of an enhanced immuno assays and of diagnostic techniques based on nucleic acid amplification.

Promotion of the use of oral fluid as viral diagnostic fluid requires that immunological assays have higher sensitivity. The development of antibody capture assays, either ^{125}I labeled (RIA) or ELISA, that are able to generate higher signals by capturing the proportion of specific to the total immunoglobulin (present in the oral fluid), enabled oral fluid to be used for successful immunological assays[97-99]. The RIA have associated problems in disposing of radioactive waste from aspect of health may decrease its acceptability. Capture ELISA is better for wide-scale use in many laboratories. A study also showed that the capture ELISA with saliva was more sensitive than the radioimmunoassay for specific rubella IgG[102]. Hence sensitivity enhancement is required to make best advantage of ELISA. This review shows that FITC/anti-FITC enhanced capture ELISA that can be used for population and vaccine surveys [68,77].

The production of measles antigen for measles diagnosis, such as the one we used for GAC- and MAC-ELISA, benefited from tissue culture. However, production of purified measles antigens in tissue culture can be difficult. The capture format has been revolutionized by the raising of purified antigen and monoclonal antibodies for use in oral-fluid measles diagnostics. Cloning and expression of measles genes provides a relatively straightforward alternative approach[99,125], simplifying purification and enabling large-scale production for improvement in measles oral-fluid diagnostic assays.

Kits based on the use of recombinant antigens such as the Light Diagnostic kit (Chemicon Temecula, CA, United States) benefited from the cloning and expression approach. More recently IgG and IgM kits specific to measles have been developed based on such an alternative approach by Microimmune Ltd (Brentford, Middlesex, United Kingdom) for both oral-fluid and serum samples. However, there are problems associated with the use of recombinant antigens associated with the production of "incorrectly" processed antigens by most expression systems[99,125] and the problem of using a single cloned antigen to detect a measles antigen that may vary between isolates. This may be resolved by cloning and expressing the most conserved region of the measles gene identified from sequence data of different isolates. Notwithstanding this problem measles antibody assays that are increasingly based on the use of cloned proteins will continue to play a prominent role in oral-fluid diagnostic development. Such immuno-assays may be useful in the future when they become better suited to use with automated systems that are capable of handling all stages of testing from specimen preparation to issuing of diagnostic results.

Microimmune assays are observed to be easy to use, but have not yet been evaluated under a wide range of conditions such as in highly vaccinated populations. The procedure and principle of the oral-fluid Microimmune EIA methodology is described in Figure 2.

Studies of rubella revealed problems of sensitivity in enhanced GACELISA in older age groups. This appears to be due to decay in the level of specific antibody in serum and in oral fluid[98,102,126]. Age-related variation in sensitivity was not seen as a big problem in measles assays[77,98]. However, low-level measles antibodies resulting from vaccine-induced immunity is a feature of many communities, particularly those with high-level routine immunizations coverage. Future work is required to evaluate the performance of newly developed kit assays in such settings.

Assays of measles nucleic acid are fundamentally different from those of measles antibodies, since they detect a component of the measles virus itself, rather than serological evidence of its past presence. Among the several techniques used to detect viral nucleic acids the PCR is the one widely used for detection of measles nucleic acid[127-129]. In contrast to direct hybridisation, whose application is restricted to where high concentration of the virus is present, PCR amplifies the probe signal by means of a sequential series of secondary, tertiary, *etc.* stages. The signal amplification thus increases the sensitivity of detection to a range where it can detect viruses at low concentration in various specimens[129]. PCR is suitable

for the detection of the low concentration of measles virus present in oral fluid. Actually oral fluid is better for nucleic acid extraction than serum or blood because of the absence of PCR inhibitors, such as haem or porphyrin, in the oral fluid[129]. In addition, oral fluid specimens do not need pre-treatment for nucleic acid extraction. In future developments of measles oral-fluid diagnosis based on the nucleic acid amplification systems are likely to play an increasing part. The new tool developed by Roche Molecular Biochemicals, MagNA Pure LC DNA isolation kit, for the isolation of nucleic acid from various types of specimen including oral fluid, is a breakthrough that has shortened the tedious manual RNA extraction process in measles nucleic acid detection. This is now practiced in many laboratories of industrialized countries but may be restricted to laboratories that have specialised requirements and too costly for most developing countries. Recently Health Protection Agency has developed point-of-care test (POCT) for the detection of measles-specific IgM antibodies in serum and oral fluid specimens and to assess if measles virus nucleic acid could be recovered from used POCT strips[123]. Further evaluation of this test method under different scenario will refine the technique for wider future application.

IgG can be measured in terms of its functional binding avidity. The binding strength between the IgG and the virus antigen is supposed to be low in primary infection and changes to high in past infection. This avidity can be measured by disrupting the interaction using protein denaturants such as urea or diethylamine[130]. Diagnosis of primary infection by IgG avidity assay using serum samples has got relevance for the diagnosis of viral infection such as rubella[131-132]. The detection of antibody with low or high avidity enables a more accurate diagnosis in differentiating primary infection from past infection.

IgG avidity is useful when the IgM assay result is indeterminate. It may also help in distinguishing primary and secondary ("boosting") response to measles vaccine. Future development of IgG avidity in oral-fluid measured by GACELISA may allow specific, sensitive and accurate diagnosis of primary infection. Our study[68] show the problem of MACELISA in detecting IgM in oral-fluid samples collected at early onset of measles rash. The future development of IgG avidity that can determine IgM in early-collected oral fluid samples makes MACELISA better use.

Another interesting area to look at in the future is the differentiation between antibodies resulting from vaccine strain and wild type measles virus. This assists in defining vaccine uptake and estimating continued measles transmission. It may be difficult to explain the technical development at this stage. However, it is an area for future research.

Evaluation of a new diagnostic test has the potential sources of bias introduced by the study design. The test's discriminatory ability, sensitivity, and specificity depend upon the composition of the study population. The study design we used for evaluation of the present measles oral- fluid diagnostic assays[68,77] is an area that can be followed in the future for other viral diagnostic test evaluation.

Future wider applications of oral-fluid methods

The application of oral-fluid methods to population surveys, vaccine surveys, diagnosis of clinical cases, and case surveillance for different vaccine uptake settings of a country/district are illustrated in Table 2. The following is a description of some of the applications of oral-fluid methods.

Population surveys: Measles antibody population surveys can used to define the proportions of susceptible and immune in the population. Population immunity may result from natural measles infection or/and measles routine and campaign vaccination. Current methods cannot distinguish between the two whether the immunity is induced by wild or vaccine virus. Population immunity surveys can identify in which age groups large pockets of susceptibles remain in unvaccinated populations, in a population with routine immunization, and before and after a vaccine campaign. This would provide valuable information on the age groups to target for vaccination and effectiveness of the routine or campaign vaccination, and clues to where future outbreaks might arise. Similarly, through such surveys hard-to-reach groups in rural/urban under different geographical settings can be reached.

Population surveys may be appropriate at all stages of vaccination programmes, in country/settings of vaccine uptake for low to high, with or without campaigns/accelerated measures. Predominantly, such surveys could assess specific antibody status. However, post-campaigns there might be a role for IgM testing in community survey to establish what proportion of the population actually responded to vaccine. Based on surveys cluster sampling techniques, as for EPI vaccine cluster sampling, and using of the-shelf EIA kits, the surveys would be rapid and simple to effect.

Vaccine surveys: Vaccine surveys assess the level of population immunity attending vaccine clinic to a measles routine vaccination. It can identify the responses to routine vaccine in pre- and post-vaccinated children. The widespread use of serological determinants of vaccine responsiveness is limited by the need to carry out follow up of vaccinees at 2 (IgM) and < 4 (IgG) wk after vaccination. Oral-fluid sampling will not improve greatly up on this situation, except that compliance for second samples is likely to be greater than if blood samples are required. However, the future development of oral-fluid IgG avidity measurement cannot be ruled out that may improve this situation.

Diagnosis: Measurement of measles antibody present in oral-fluid samples provides information on the status of current and past infection by use of tests for IgG and IgM antibody. Laboratory diagnosis of suspected measles clinical cases can assist in (1) confirmation of the occurrence of measles clinical illness (2) capability of physicians to diagnose illness and (3) reporting of the infection to health department. The usefulness of

Table 2 Application of oral-fluid methods under different countries settings

Applications of oral-fluid methods	Setting for country/district		
	Low/Med uptake routine	High uptake routine	Campaign
Population survey	Methods: community surveys of IgG across wide age range. Including hard-to-reach groups, informal settlements. Purpose: immunity profiles. Identifies susceptibility gaps and age range for campaigns. Implications: increase in coverage, need for and age range for campaigns	As previous	As previous plus. Methods: Post-campaign surveys of IgG and perhaps IgM. Purpose: IgG-Identify immunity levels post-campaign. Susceptibility in target age group and outside target group. IgM-indicator of impact, *i.e.*, proportion responding to vaccine. Implications: Age-range for future campaigns; locate problems of vaccine efficacy
Vaccine surveys	Methods: Vaccine clinic samples pre- and post-vaccination. IgM and/or IgG testing. Purpose: Assess efficacy of routine vaccination. Implications: Identify cause of low efficacy.	As previous	As previous plus. Methods: IgG survey of individuals attending vaccine clinics. Purpose: Identify proportion able to respond to vaccine. Implications: Assess potential effectiveness, and suggest alternative method for delivery eg hard-to-reach groups.
Diagnosis	Not indicated while measles incidence remains high	Method: IgM testing on demand. Purpose: Confirmation of clinical diagnosis	As previous
Case surveillance: serological and genetic	Not indicated while measles transmission remains high	Method: System of reporting and oral fluid sampling from sporadic cases and outbreaks. IgM and Genotyping Purpose: Verify cases, and monitor distribution of virus and endemicity Implications: Need for additional control measures	As previous

IgG: Immunoglobulin G; IgM: Immunoglobulin M.

oral fluid in this capacity is at present hindered by the relatively low sensitivity of IgM assays in samples taken early after onset of rash. A study showed the oral fluid measles IgM detection rate increased from 63%-67% at 2 d and 3%-100% at days 6 and 7[82]. Delay in collecting a sample may be impractical. Improved sensitivity of assays remains a need.

Case surveillance: The recognition and identification of measles outbreaks and sporadic cases using a system of reporting and oral-fluid sampling is established in the United Kingdom[20,76,133]. For measles epidemic investigation in Ethiopia, where infrastructure is poor and locations of the remote, oral-fluid sampling was found to be appropriate. Especially in the situations where community beliefs or attitudes like "measles sick should not get injection" are present, in which communities declined to give blood specimens, oral-fluid specimens are preferable. Provided reasonable storage conditions while in transit or awaiting transit to the laboratory are made, oral-fluid is a robust sample for IgG testing, IgM testing and viral genome detection (United Kingdom surveillance and in these studies in Ethiopia)[77,134]. However, further stability studies of oral-fluid at different temperature in field conditions are required in the future.

Another area of increasing importance is the application of sequence data obtained from oral-fluid nucleic acid amplification techniques. Genetic information is valuable, in combination with other traditional epidemiological data, to enhance the ability to determine measles transmission pathways and to assess the success of measles control strategies[79,124,135,136].

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ORIGINAL ARTICLE

Epigallocatechin-3-gallate suppresses transforming growth factor-beta signaling by interacting with the transforming growth factor-beta type Ⅱ receptor

Masaki Tabuchi, Sumio Hayakawa, Eiko Honda, Kana Ooshima, Tatsuki Itoh, Koji Yoshida, Ah-Mee Park, Hideaki Higashino, Mamoru Isemura, Hiroshi Munakata

Masaki Tabuchi, Koji Yoshida, Ah-Mee Park, Hiroshi Munakata, Department of Biochemistry, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan
Sumio Hayakawa, Division of Signaling in Cancer and Immunology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan
Eiko Honda, Life Science Research Institute, Kinki University, Osaka-Sayama 589-8511, Japan
Kana Ooshima, Hideaki Higashino, Department of Pharmacology, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan
Tatsuki Itoh, Department of Pathology, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan
Mamoru Isemura, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
Author contributions: Tabuchi M and Hayakawa S contributed equally to this work; Tabuchi M and Hayakawa S designed studies; Tabuchi M, Hayakawa S, Honda E, Ooshima K, Itoh T, Yoshida K, Park AM, Higashino H and Isemura M performed the research; Tabuchi M, Hayakawa S and Munakata H analyzed the data; Munakata H wrote the paper.
Correspondence to: Hiroshi Munakata, Professor, Department of Biochemistry, Kinki University Faculty of Medicine, 377-2 Ohnohigashi, Osaka-Sayama 589-8511, Japan. munakata@med.kindai.ac.jp
Telephone: +81-72-3660221 Fax: +81-72-3660245
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Abstract

AIM: To investigate the (-)-epigallocatechin-3-gallate (EGCG) binding to transforming growth factor-β (TGF-β) type Ⅱ receptor (TGFRⅡ).

METHODS: The expression of α-smooth muscle actin (α-SMA) was used as a marker for fibrotic change in human lung fibroblast MRC-5 cells. The α-SMA expression level was determined by western blotting and immunohistological analysis. We examined whether the anti-fibrotic effects of EGCG on MRC-5 cells was dependent on antioxidant mechanism by using edaravone and *N*-acetylcysteine (NAC). The suppression effects of EGCG on Smad2/3 activation were studied by confocal fluorescence microscopy. The binding of EGCG to recombinant TGFRⅡ protein was analyzed by immunoprecipitation and affinity chromatography.

RESULTS: When MRC-5 cells were treated with TGF-β, EGCG decreased the expression of α-SMA in a dose dependent manner, whereas catechin did not influence the α-SMA expression in the cells. Except for EGCG, antioxidant compounds (*e.g.,* edaravone and NAC) had no effects on the TGF-β-induced α-SMA expression. Nuclear localization of phosphorylated Smad2/3 was observed after TGF-β treatment; however, EGCG treatment attenuated the nuclear transportation of Smad2/3 in the presence or absence of TGF-β. After a TGFRⅡ expression vector was introduced into COS-7 cells, cell lysates were untreated or treated with EGCG or catechin. The immunoprecipitation experiments using the lysates showed that EGCG dose-dependently bound to TGFRⅡ and that catechin did not at all. Affinity chromatography study indicated that EGCG would bind to TGFRⅡ.

CONCLUSION: Our results demonstrate that EGCG interacts with TGFRⅡ and inhibits the expression of α-SMA via the TGF-β-Smad2/3 pathway in human lung fibroblast MRC-5 cells.

Key words: Epigallocatechin-3-gallate; Transforming

growth factor-β; Myofibroblast; α-smooth muscle actin; Fibrosis

Core tip: (-)-Epigallocatechin-3-gallate (EGCG) binds to transforming growth factor-β (TGF-β) type Ⅱ receptor (TGFRⅡ) and inhibits TGF-β action by interfering with the interaction between TGF-β and TGFRⅡ. Because TGF-β is considered to be the strongest inducer of tissue fibrosis, the obtained data from this investigation suggest that EGCG may be a new therapeutic agent for organ fibrosis.

Tabuchi M, Hayakawa S, Honda E, Ooshima K, Itoh T, Yoshida K, Park AM, Higashino H, Isemura M, Munakata H. Epigallocatechin-3-gallate suppresses transforming growth factor-beta signaling by interacting with the transforming growth factor-beta type II receptor. *World J Exp Med* 2013; 3(4): 100-107 Available from: URL: http://www.wjgnet.com/2220-315X/full/v3/i4/100.htm DOI: http://dx.doi.org/10.5493/wjem.v3.i4.100

INTRODUCTION

(-)-epigallocatechin-3-gallate (EGCG), the most biologically active constituent in green tea, has been recognized as a component that provides the beverage with potential benefits for human health[1]. The reported health-promoting properties of green tea include anti-cancer[1-3], anti-obesity[4], anti-diabetic[5,6], anti-atherosclerotic[7], anti-viral[8-10], anti-bacterial[11-13] and neuroprotective[14-16] effects. The anti-fibrotic effects of green tea and its constituents, especially EGCG, on liver fibrosis[17-19], pancreatic fibrosis[20] and pulmonary fibrosis[21] have been also reported.

Activation of myofibroblasts is the one of the critical events during fibrosis development. Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that is pivotal in the regulation of myofibroblast activation, differentiation, migration, and extracellular matrix production; it also plays an important role in the initiation and progression of fibrosis[22]. However, the mechanisms by which EGCG influences TGF-β action on myofibroblast activation remain incompletely defined.

Tachibana *et al*[23] identified a catechin receptor for EGCG, and showed that this receptor partially mediates the function of EGCG. It is also known that EGCG shows its biological action by interacting with receptors other than the catechin receptor[24,25]. In the present study, we investigated the possibility that EGCG might bind to the TGF-β type Ⅱ receptor (TGFRⅡ).

MATERIALS AND METHODS

Cell culture

The MRC-5 and COS-7 cell lines were obtained from the Riken Cell Bank (Tsukuba, Japan), and were maintained in Dullbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, United States) at 37 ℃ under 5% carbon dioxide and 95% air.

Chemicals

Catechin and EGCG were obtained from Funakoshi Co. (Tokyo, Japan) and dissolved in PBS. *N*-acetylcysteine (NAC) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and dissolved in dimethyl sulfoxide. Edaravone was the product of Mitsubishi Tanabe Pharma (Osaka, Japan). TGF-β was obtained from R&D Systems (Minneapolis, MN, United States).

Antibodies

The following antibodies were used in this study: monoclonal anti-FLAG antibody produced in mouse (anti-Flag) (Sigma); monoclonal anti-α-smooth muscle actin antibody (anti-α-SMA) produced in mouse (Sigma); monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (anti-GAPDH) produced in mouse (Sigma); rabbit anti-Smad2/3 antibody (anti-Smad2/3) (Cell Signaling Technology, Danvers, MA, United States); and goat anti-human TGFRⅡ antibody (anti-TGFRⅡ), which recognizes extracellular domain of the receptor (R and D).

Western blotting

After washing with ice-cold phosphate buffer saline (PBS), cells were treated with 0.25% trypsin-EDTA solution (Invitrogen, Carlsbad, CA, United States), suspended in growth medium and collected by centrifugation at 700 *g* for 5 min. The pellets were washed with PBS, resuspended in lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate), which contained a cocktail of protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany) on ice, and centrifuged at 18000 *g* at 4 ℃ for 10 min.

Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, United States). Cell lysates were suspended in SDS electrophoresis sample buffer and boiled for 5 min. Samples (2.5 μg of protein per lane) were separated on 10% polyacrylamide gels and then transferred to an Immobilon P membrane (Millipore, Billerica, MA, United States). Antibody binding was detected by ECL Plus (GE Healthcare, Buckinghamshire, United Kingdom).

Immunohistological studies

Cells were seeded on BD Falcon 8-well CultureSlide. Cells were cultured under indicated conditions. Medium was removed, and cells were washed with PBS, fixed by 3% paraformaldehyde in PBS for 10 min. After washing with PBS, cells were permeabilized by 0.1% Triton X-100 in PBS. Fixed cells were sequentially treated with anti-α-smooth muscle actin (SMA) antibody (1/100, 37 ℃, 1 h), and fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin G (37 ℃, 30 min). Actin stress fibers were visualized by rhodamine-labeled phalloidin (1/50,

37 ℃, 10 min). For staining the nuclei, cells were treated with 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 20 min. Cells were examined with a fluorescence microscope (Nikon ECLIPSE E-800, Nikon Corporation, Tokyo, Japan) equipped with a fluoresecence digital microscope camera controller (VB-7000; Keyence Co., Osaka, Japan).

Plasmid construction

Plasmid was constructed according to standard recombinant DNA techniques. The fragment encoding the human TGFR Ⅱ cDNA (Met1-Lys567, GenBank accession no. M85079) was amplified from a human fetal liver cDNA library (OriGene Technologies, Rockville, MD, United States) by polymerase chain reaction (PCR) with KOD Plus DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) using the primers 5'-TTTGAATTCGCCATGGGTCGGGGGCTGCTC-3' (forward) and 5'-TTTGGATCCTTTGGTAGTGTTTAGGGAGCC-3' (reverse). The forward and reverse primers were designed to introduce an *Eco*R Ⅰ and a *Bam*H Ⅰ restriction site (underlined), respectively, for subcloning purposes. The PCR product was cloned into the pFlag-CMV-5a vector (Sigma). The construct was verified by DNA sequencing.

Transfection

COS-7 cells, grown to 50%-70% confluence, were transfected using Lipofectamine plus (Invitrogen) according to the manufacturer's instructions. The transfectants were grown in DMEM containing 10% FBS. After 3 d, the medium was removed and expression of TGFR Ⅱ in the cells was examined by western blotting.

Immunoprecipitation

Cell lysates were treated with Protein G Sepharose (GE Healthcare) for 30 min at 4 ℃ to remove proteins nonspecifically bound to Protein G Sepharose. Anti- TGFR Ⅱ antibody was then added to the above lysate, and incubated for 2 h at 4 ℃. Next, Protein G Sepharose was added and incubated for 1 h at 4 ℃. Protein G Sepharose was recovered by centrifugation and washed three times with PBS. The immunoprecipitated proteins were removed from the Protein G Sepharose by boiling in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 min and then separated by electrophoresis.

Affinity chromatography

EGCG was coupled to CNBr-activated Sepharose 4B (GE Healthcare) at a concentration of 5 mg/mL of wet gel. Cell lysate was applied to a column of EGCG-Sepharose 4B and washed with PBS. Bound proteins were eluted with 4 mol/L urea, 1 mol/L NaCl in PBS, and fractions of 0.25 mL were collected. An aliquot of each fraction was spotted onto polyvinylidene difluoride (PVDF) membrane and stained with Coomassie Brilliant Blue. A portion of each fraction was also examined by western blot analysis after SDS-PAGE using anti-TGFR Ⅱ antibody.

RESULTS

Effects of EGCG on the expression of α-smooth muscle actin

The MRC-5 cell line, which is derived from human fetal lung fibroblasts, expresses α-SMA and is considered to be a myofibroblast cell line[26,27]. Therefore, this cell line was used in this study.

MRC-5 cells were grown to 85% confluence, and then serum-starved (0.5% FBS) for 48 h. After serum starvation, cells were treated with TGF-β. We and others usually use 1-2 ng/mL of TGF-β in culture media[27-31]. A representative and frequently used marker of myofibroblast activation is α-SMA[32,33]. Western blot analysis and immunohistological examination showed that expression of α-SMA was increased by TGF-β (Figure 1). Whereas a catechin control showed no effects on α-SMA expression, EGCG dose-dependently abolished the increase in expression of α-SMA induced by TGF-β (Figure 1B). The EGCG concentration used in this study was reasonable[34]. The expression of GAPDH also seemed to be decreased by a high dose of EGCG. The band densities of α-SMA and GAPDH were compared (Figure 1B), and the result clearly showed the effects of EGCG on α-SMA.

Because EGCG is an antioxidant compound, we examined whether edaravone and NAC, two well-known antioxidant compounds, have similar effects. Neither treatment with edaravone (Figure 2A) nor treatment with NAC (Figure 2B) affected the increase in expression of α-SMA induced by TGF-β.

EGCG suppresses SMAD activation

The effects of TGF-β are largely mediated by Smad proteins. TGF-β causes phosphorylation of Smad2/3, and then phosphorylated Smads enter into the nucleus. After treatment with TGF-β, MRC-5 cells were examined by confocal fluorescence microscopy. Nuclear localization of phosphorylated Smad2/3 was observed after TGF-β treatment, whereas EGCG treatment clearly decreased the nuclear transportation of Smad2/3 (Figure 3).

EGCG binds to TGFR Ⅱ

Next, we examined the possibility that EGCG interferes with binding of TGF-β to the TGFR Ⅱ. To this end, cells expressing large amounts of the receptor are preferable. Because COS-7 cells showed high transformation efficiency and marked expression of exogenous cDNA, these cells were used for transformation experiments. A TGFR Ⅱ expression vector was introduced into COS-7 cells. Cell lysates were untreated or treated with EGCG or catechin, and then subjected to immunoprecipitation with anti-TGFR Ⅱ antibody. In untreated lysate and lysate treated with catechin, TGFR Ⅱ was precipitated by the antibody. When lysate was treated with EGCG, however, anti-TGFR did not precipitate TGFR Ⅱ (Figure 4).

To confirm the binding of EGCG to TGFR Ⅱ, we next performed affinity chromatography. Namely, cell ly-

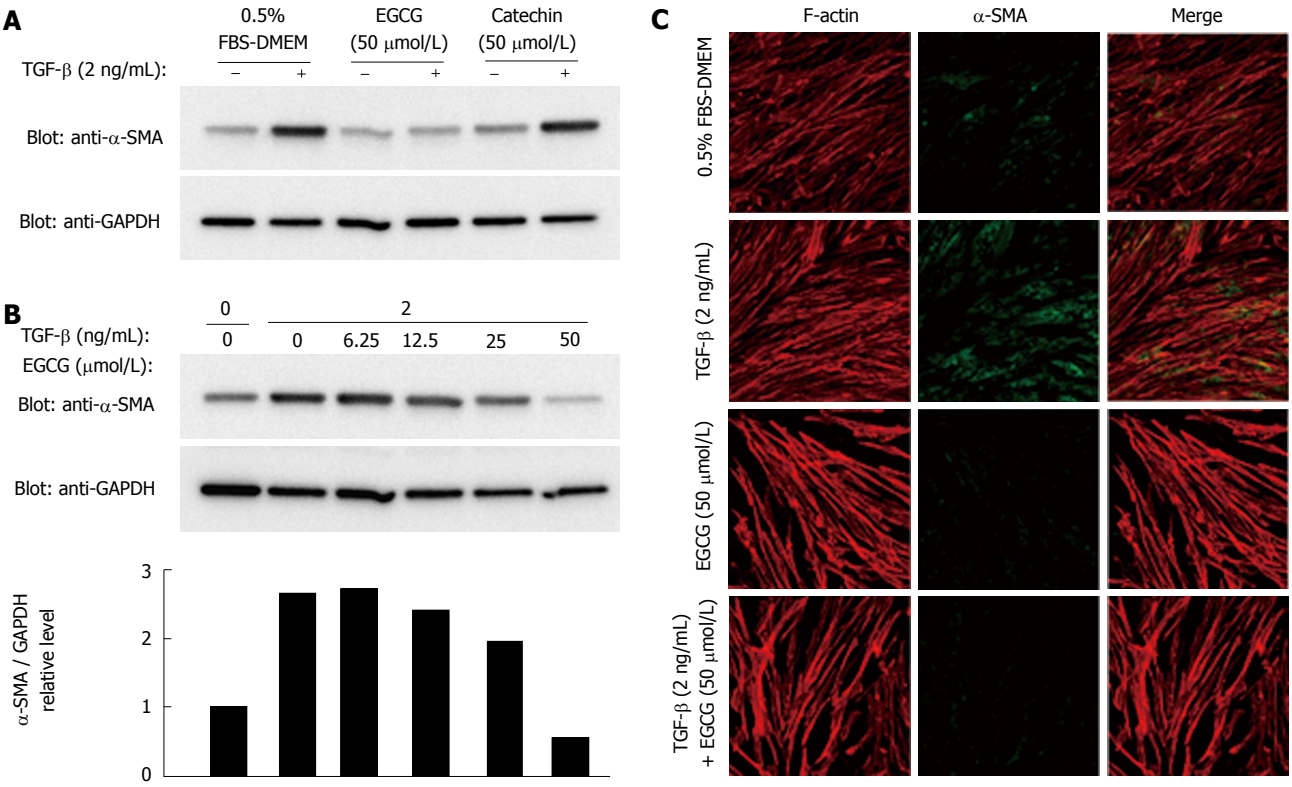

Figure 1 Effects of (-)-epigallocatechin-3-gallate on expression of α-smooth muscle actin. A: Lysates of MRC-5 cells were obtained from cells treated with 0.5% FBS in DMEM alone, (-)-epigallocatechin-3-gallate (EGCG) (50 μmol/L), or catechin (50 μmol/L) for 24 h. After SDS-PAGE, proteins were blotted onto Immobilon, and probed with anti-α-smooth muscle actin (α-SMA) antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control; B: MRC-5 cells were treated with the indicated amounts of EGCG. α-SMA was detected in the same manner as in (A). The expression levels of α-SMA were normalized to those of GAPDH; C: Expression of α-SMA in cells treated with EGCG (50 μmol/L) in the absence (-) or presence (+) of transforming growth factor-β (TGF-β) (2 ng/mL) were examined by confocal microscopy. Green: α-SMA (fluorescein isothiocyanate conjugated goat anti-mouse IgG); Red: Actin stress fiber (rhodamine-labeled phalloidin); blue: Nuclei (DAPI). FBS: fetal bovine serum.

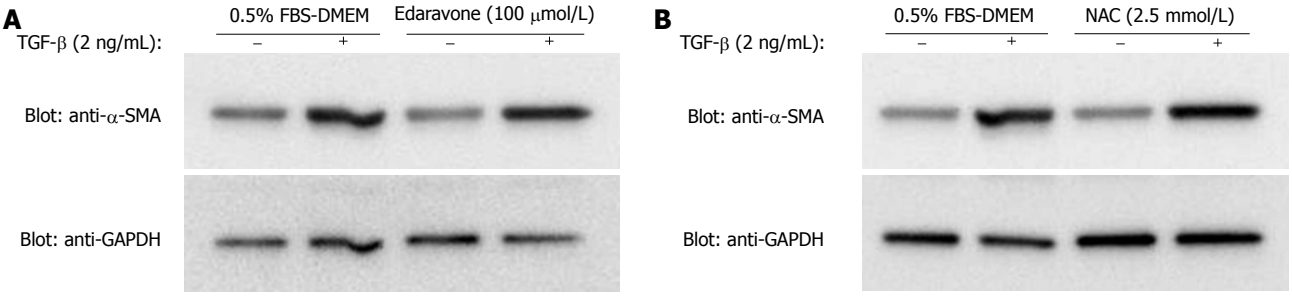

Figure 2 Effects of scavenging compounds on expression of α-smooth muscle actin. MRC-5 cells were treated with edaravone (100 μmol/L) (A) or *N*-acetylcysteine (NAC) (2.5 mmol/L) (B) for 1 h, and then stimulated with transforming growth factor-β (TGF-β) for 24 h. Cell lysates were electrophoresed, blotted onto Immobilon, and probed with anti-α-smooth muscle actin (α-SMA). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. FBS: Fetal bovine serum.

sates were applied to an EGCG-conjugated agarose column and proteins bound to the column were examined by western blotting. Figure 5 shows that TGFR Ⅱ bound to the column, indicating that EGCG binds to TGFR Ⅱ.

DISCUSSION

In this study, we have demonstrated that EGCG both inhibits the signal transduction of TGF-β by binding to TGFR Ⅱ and attenuates the expression of α-SMA in MRC-5 cells, which is a myofibroblast cell line, when it is stimulated by TGF-β. Myofibroblasts play crucial roles in the pathogenesis of tissue fibrosis[35]. Stimulation by TGF-β and other cytokines leads myofibroblasts to an activated state[36]. Activated myofibroblasts then secrete collagen and other components of the extracellular matrix, which can result in fibrosis[37].

TGF-β is the most potent cytokine causing fibrosis. Both Smad-dependent and Smad-independent TGF-β signaling pathways are known. Initiation of both pathways takes place via binding of TGF-β to its receptor. TGF-β binds to a type Ⅱ receptor, which then phosphorylates a TGF-β type I receptor. Subsequently, the type I receptor phosphorylates R-Smads (receptor-

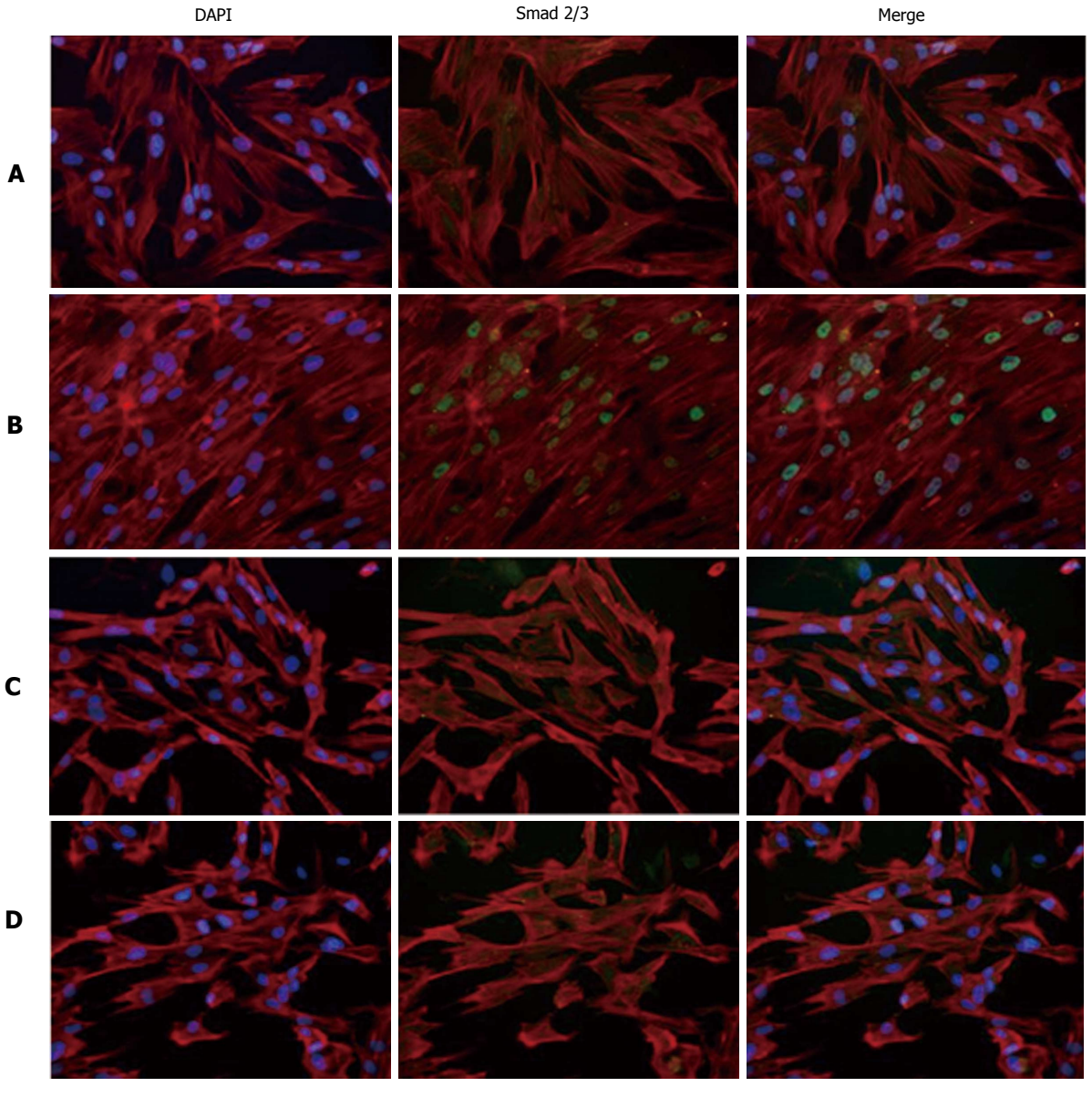

Figure 3 Effects of (-)-epigallocatechin-3-gallate on activation and localization of Smad2/3. MRC-5 cells were treated with transforming growth factor-β (TGF-β) and/or (-)-epigallocatechin-3-gallate (EGCG). Cells were examined by confocal microscopy. Subcellular localization of Smad2/3 (green) and actin stress fibers (red) are shown. Nuclei were stained by DAPI (blue). A: Control; B: Treated with TGF-β; C: Treated with EGCG; D: Treated with TGF-β and EGCG. DAPI: 4',6-diamidino-2-phenylindole.

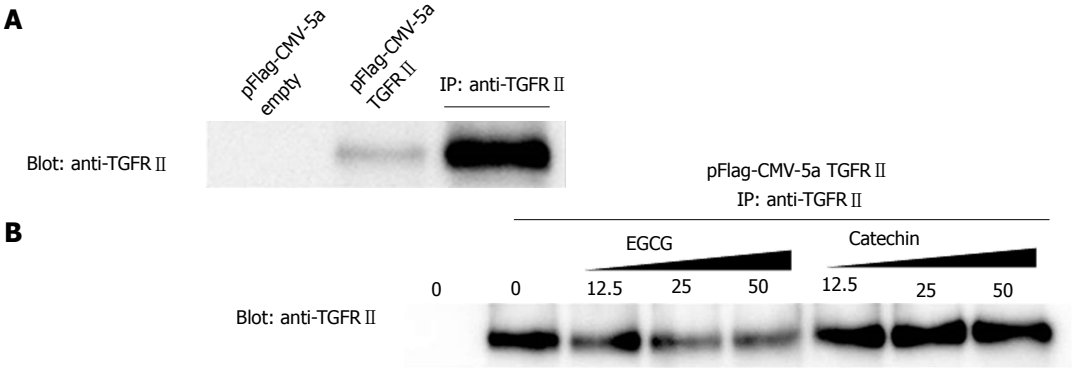

Figure 4 (-)-Epigallocatechin-3-gallate interferes with binding between transforming growth factor-β and its type Ⅱ receptor. A: Positive control of the immunoprecipitation experiment. Cell lysates from transfected COS-7 cells were treated with anti-transforming growth factor-β type Ⅱ receptor (TGFR Ⅱ). TGFR Ⅱ was recovered in the immunoprecipitation product of the lysate; B: Effects of (-)-epigallocatechin-3-gallate (EGCG) and catechin on the antigen-antibody interaction. After cells were treated with EGCG or catechin, anti-TGFR Ⅱ bound to Protein G was added to each lysate. Western blotting was performed using anti-TGFR Ⅱ.

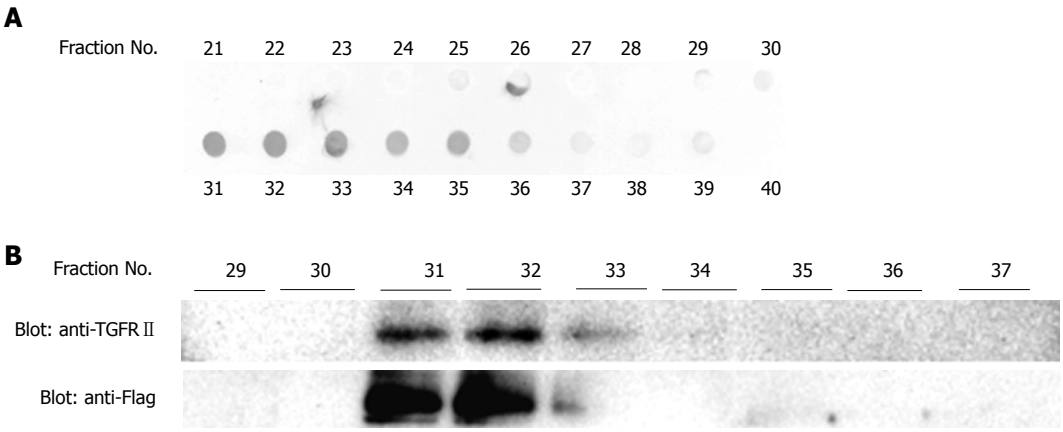

Figure 5 Transforming growth factor-β type Ⅱ receptor binds to (-)-epigallocatechin-3-gallate. A: Proteins in the fractions were detected by staining with Coomassie Brilliant Blue. An aliquot of each fraction was blotted on polyvinylidene difluoride membrane and stained; B: The protein-containing fractions revealed in (A) were electrophoresed and Western blotting was performed using anti-Flag antibody. TGFR Ⅱ: Transforming growth factor-β type Ⅱ receptor.

regulated Smads), and phosphorylated R-Smads bind to Co-Smad (common-mediator Smad). R-Smad/Co-Smad complexes translocate into the nucleus, where they act as transcription factors[38]. In this manner, regulation of TGF-β target gene expression is carried out. Expression of many proteins in MRC-5 cells changes after stimulation by TGF-β[39]. A frequently used marker of the activation of myofibroblast is α-SMA; therefore, this protein was also used as a marker in this study. Expression changes after TGF-β stimulation in cells other than MRC-5 has been observed, for example, in IMR-90 human lung fibroblasts[33] and WI38-VA13 cells[40]. Besides α-SMA, upregulation of collagen I [41], fibronectin[27]and CTGF[42] has been reported when human lung fibroblasts are treated with TGF-β.

The expression of α-SMA is regulated by Smad[43]. TGF-β increases the nuclear translocation of Smad and expression of α-SMA. We examined the influence of EGCG treatment on Smad2/3 appearance in MRC-5 cells. Immunohistological experiments indicated that EGCG inhibits the nuclear transportation of Smad2/3.

Moreover, we found that EGCG had suppressive effects on the expression of α-SMA in MRC-5 cells, whereas catechin did not. These data suggest that the effects are dependent on the gallate or pyrogallol moiety of EGCG.

Next, we investigated the mechanism of the inhibitory effect on the Smad2/3 pathway. EGCG is a potent antioxidant and a lot of its health benefit effects are thought to be due to its antioxidative action[44-46]. EGCG attenuated the increase in α-SMA expression brought about by TGF-β, whereas edaravone and NAC did not. These results indicate that the inhibitory effect of EGCG on α-SMA expression is independent of its antioxidative action.

We thought that part of the EGCG's effects on α-SMA expression might arise through interference with receptor-ligand binding. Indeed, EGCG treated cell lysate containing TGFR Ⅱ showed no immunoprecipitation with anti-TGFR Ⅱ antibody. The interaction between EGCG and TGFR Ⅱ was also confirmed by the affinity chromatography experiment. A likely explanation for this observation is that EGCG binds to TGFR Ⅱ, thereby blocking the antibody from binding to TGFR Ⅱ. Similarly, if EGCG binds to the TGF-β receptor, TGF-β would not be able to bind to its receptor and downstream signaling pathways would be ineffective.

In conclusion, we have shown that EGCG interacts with TGFRII and inhibits the expression of α-SMA via the TGF-β-Smad2/3 pathway in MRC-5 cells, which are human lung fibroblasts. These results suggest that EGCG has anti-fibrotic effects that are crucial for the control of myofibroblast differentiation and extracellular matrix deposition, which are involved in fibrosis. The evidence that EGCG is effective in the suppression of fibrosis may lead not only to better understanding of the biological roles of EGCG but also to clinical applications of this flavonoid.

COMMENTS

Background

Fibrosis is an intractable disease. Effective treatments for it have not been developed yet. Catechin is a substance with a variety of physiological effects. However, the investigation on the antifibrotic effect of catechin has not been fully performed.

Research frontiers

Various physiological effects of catechin have been intensely studied. It has been reported that catechin has a variety of physiological activity (*e.g.*, regulation of blood pressure, blood cholesterol, blood sugar; antioxidant, anti-aging, anti-cancer effects).

Innovations and breakthroughs

Many studies have been performed about (-)-epigallocatechin-3-gallate (EGCG) relationship with transforming growth factor-β (TGF-β) and its antifibrotic properties. We demonstrated that EGCG inhibits the TGF-β activity through its binding to TGF-β type Ⅱ receptor (TGFR Ⅱ).

Applications

TGF-β is believed to be the strongest inducer of tissue fibrosis. EGCG inhibits TGF-β activity by interacting with TGFR Ⅱ. Therefore, EGCG may become an antifibrotic agent.

Terminology

Green tea contains four main catechin substances: Epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, all of which are

inclusively called catechin. Organ fibrosis is a clinical condition caused by an excessive deposition of extracellular matrix. The progression of fibrosis resulted in a loss of normal function.

Peer review

This paper reports a novel, interesting and important study. This is a basic work which shows that EGCG could bind to the TGFRⅡ abolishing myofibroblast activation. The original point in this work is the analysis that is done on the cytokine receptor. The authors soundly demonstrated the binding EGCG to TGFRⅡ by immunoprecipitation and affinity chromatography experiments.

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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

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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]

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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]

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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

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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

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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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