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Platelet-derived growth factor in middle ear cholesteatoma

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Abstract Platelet-derived growth factor (PDGF) was localized in human middle ear cholesteatoma tissue by an immunoperoxidase technique using rabbit anti-human PDGF IgG. PDGF was found mainly in basal cells and in granulation tissue, and especially involved monocytes and fibroblast-like cells. The external ear canal epithelium was not significantly stained by anti-human PDGF. Findings demonstrate that the presence of PDGF in cholesteatoma is in response to inflammation and wound healing in the middle ear. PDGF in vitro was found to stimulate protein synthesis and cellular terminal differentiation of basal keratinocytes. PDGF also stimulated monocytes to form multinucleated osteoclast-like cells. These multinucleated cells, in turn, induced the resorption of devitalized bovine bone. This bone resorption was seen in co-cultures of osteoblasts and multinucleated osteoclast-like cells in the presence of PDGF, suggesting that cell-to-cell interaction plays a role in bone resorption. The present study suggests that PDGF takes part in the clinical development and the destructive effect of cholesteatoma.

Key words Cholesteatoma · Platelet-derived growth factor · Keratinocyte · Bone resorption

Introduction

Platelet-derived growth factor (PDGF) is a polypeptide that was originally found in platelets with a molecular weight of 30,000 daltons. To date, many different types of cells can produce PDGF in the inflammatory response, including activated macrophages, endothelial cells, epithelial cells, and fibroblasts [17]. It has also been shown that PDGF plays a role in wound-healing activities by stimulating neutrophils, monocytes, and fibroblasts for proliferation and chemotaxis [5, 6, 15, 21]. Recently, it has been reported that PDGF enhances bone resorption by increasing synthesis of collagenase and prostaglandin E$_2$ (PGE$_2$) [13, 20].

Bone resorption and progressive growth of a squamous epithelium are pathologic features of middle ear cholesteatoma. We have reported previously that several factors, such as granulocyte-macrophage colony stimulating factor (GM-CSF) [9], interleukin 1 (IL-1) [2, 3], and tumor necrosis factor-α (TNF-α) [24, 25] stimulate bone resorption and proliferation as well as cellular differentiation of keratinocytes. This proliferation and differentiation of keratinizing epidermal cells appears to be a crucial factor in the pathogenesis of middle ear cholesteatoma. We have also reported that the conditioned medium of inflammatory connective tissue stimulates proliferation and differentiation of epidermal basal cells [8]. The stimulatory effects of this conditioned medium are due to its various factors, including PGE$_2$, epidermal growth factor, and IL-1. Since acquired cholesteatoma is associated with an inflammatory reaction, we wanted to determine if PDGF is also involved in the disease process of cholesteatoma. In this study, we attempted to demonstrate the distribution of PDGF in human middle ear cholesteatoma and the effects of PDGF on the proliferation, protein synthesis and terminal differentiation of basal keratinocytes, and bone resorption in order to delineate a possible role of PDGF in the pathogenesis of cholesteatoma.

Materials and methods

Human middle ear cholesteatoma tissues (ten specimens) and normal external auditory canal skins (five specimens) were obtained from patients during ear surgery. The ages of the patients ranged from 21 to 56 years, with a median age of 42. The protocols for using specimens for this study were approved by the Columbia University Institutional Human Use Committee Review Board and the studies were in compliance with all United States federal, state and local regulations concerning the use of human subjects and materi-
als in research. Tissue specimens removed were fixed in phosphate-buffered 10% formaldehyde solution, embedded in paraffin, and sectioned in 6 μm thickness.

Immunohistochemical staining

Tissue sections were stained by the indirect immunoperoxidase method. The procedure of immunoperoxidase staining included the following incubation steps: (1) 3% hydrogen peroxide, 5 min; (2) normal goat serum (Cappel, Cochranville, Pa.), diluted 1:30, 20 min; (3) rabbit anti-human PDGF IgG, 25 μg/ml (R & D Systems, Minneapolis, Minn.); (4) peroxidase-conjugated goat anti-rabbit IgG, 10 μg/ml, 30 min (Sigma, St. Louis, Mo.); and (5) 0.06% 3, 3'-diaminobenzidine-0.003% hydrogen peroxide in phosphate-buffered saline (PBS), 10 min. For control stainings, PBS or non-immunized whole IgG or antibody absorbed with PDGF was used instead of primary antibody.

Preparation of primary cell cultures

Basal keratinocytes were prepared from the skin of newborn rats, as described previously [19]. Monocytes were obtained from the narrow cavities of adult rat femurs and tibiae. The contents of the narrow cavities were expelled with Dulbecco’s modified Eagle medium (DMEM) and incubated at 37°C in 95% air-5% carbon dioxide for 2 h. Non-adherent cells were discarded. More than 95% of the adhered cells were monocytes and demonstrated non-specific esterase activity, a marker enzyme for monocytes [3].

Osteoblasts were prepared from calvaria of newborn rats. Calvaria were cleaned to remove overlying soft tissue and periosteum, and digested with collagenase (2 mg/ml) (Sigma) at 37°C, for 20 min. This procedure was repeated four times. The cells from the third and fourth digestions were cultured in DMEM with 10% fetal bovine serum (FBS). More than 95% cells prepared were osteoblasts with positive alkaline phosphatase activity [3].

Incorporation of 3H-thymidine and 3H-histidine and 3H-putrescine

Cell proliferation and protein synthesis were determined by the incorporation of 3H-thymidine and 3H-histidine into basal keratinocytes. Basal keratinocyte cultures (3 x 10⁵ cells/well) were placed in F-10 medium plus 10% FBS at 37°C in 95% air-5% carbon dioxide. When the keratinocyte culture neared confluence, the medium was replaced with DMEM without FBS. Recombinant human PDGF-BB (0-30 ng/ml) (Genzyme, Boston, Mass.) was added and incubated for 48 h. Basal keratinocytes were then labeled with 3H-thymidine or 3H-histidine (0.5 μCi/well) and incubated for another 24 h. Incorporation of 3H-thymidine and 3H-histidine was determined by counting radioactivity in the cells after treatment with 10% trichloroacetic acid and being dissolved in 1 N sodium hydroxide.

Since transglutaminase is an enzyme that stimulates formation of isopeptide cross-linking to form insoluble protein during cellular terminal differentiation (cornification) of keratinocytes, its activity in basal keratinocytes was determined by measuring incorporation of 3H-thymidine and 3H-histidine into keratinocyte cultures after 48 h treatment with various concentrations of recombinant PDGF-BB. Incorporation of 3H-thymidine into insoluble cell envelopes was then determined by a scintillation counter. In some experiments, anti-PDGF or 5 mM cystamine was added to inhibit transglutaminase activity.

Isolation of cornified cell envelopes

Cornified cell envelopes formed during terminal differentiation of keratinocytes were isolated by the procedure of King et al. [10]. Keratinocytes were detached from culture plates after 10 min treatment with 0.05% trypsin-0.53 mM EDTA. Isolated cells were then treated with 200 μM calcium ionophore A 23187 (Sigma). After incubation at 37°C for 2 h, cells were lysed by the addition of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol at a final concentration of 2%. For counting cell envelopes, 10 μl of deoxyribonuclease (1 mg/ml) was added to the cell lysate to prevent the aggregation of cell envelopes. Cornified cell envelopes were counted under a microscope using a hemocytometer. The degree of

Fig. 1A–C Indirect immunoperoxidase staining of platelet-derived growth factor (PDGF) in cholesteatoma. A Positive brownish staining can be seen in basal cells (B) of the epidermal layer. B In the subepithelial granulation tissue, PDGF is localized in monocytes/macrophages (M) and fibroblast-like cells (F). C Control study of indirect immunoperoxidase staining of PDGF in cholesteatoma (absorption test). No significant staining is seen in either the epithelial layer (E) and granulation tissue (G). Original magnification, × 240.