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Epidermal growth factor receptor inhibition by erlotinib prevents vascular smooth muscle cell and monocyte–macrophage function in vitro

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ABSTRACT

Introduction: Vascular smooth muscle cells (VSMCs) and monocyte–macrophages play a central role during the development of chronic allograft injury, which still remains an important challenge in organ transplantation. Inflammation, fibrosis and accelerated arteriosclerosis are typical features for chronic allograft injury. Growth factors participate in cell proliferation, differentiation and migration in this pathological process.
Objective: Here we studied the role of epidermal growth factor receptor (EGFR) in VSMC and monocyte–macrophage function in vitro. EGFR inhibition by erlotinib, a selective EGF tyrosine kinase inhibitor, was studied in VSMC proliferation and migration as well as monocyte–macrophage proliferation and differentiation.
Materials and methods: Rat coronary artery SMCs were used for VSMC studies. As a model for monocyte–macrophage proliferation and differentiation human monocytic cell line U937 was used. Phorbol ester TPA was used to induce these cells to differentiate into macrophages.
Results: Platelet-derived growth factor (PDGF)-B, a known VSMC inducer, caused 2.1-fold stimulation in VSMC proliferation compared to non-stimulated VSMC. Erlotinib prevented this VSMC proliferation in a dose-dependent manner, $p < 0.001$ in all groups compared to controls. PDGF-B stimulation increased VSMC migration to 2.5-fold when compared with non-stimulated cells. Erlotinib decreased VSMC migration dose-dependently and this effect was significant with all doses, $p < 0.05$. Erlotinib inhibited dose-dependently the proliferation of U937 monocytic cells, $p < 0.001$. Erlotinib prevented also TPA-induced macrophage differentiation in a dose-dependent way, $p < 0.05$.
Discussion: Erlotinib significantly prevents VSMC proliferation and migration in vitro. Erlotinib inhibited also significantly both monocyte proliferation and differentiation. Our data suggest that EGFR inhibition in VSMC and monocyte function has beneficial effects on chronic allograft injury.

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1. Introduction

Chronic allograft injury still remains an important challenge in organ transplantation [1,2]. Currently there is no treatment for this injury, which leads to declining graft function and subsequent graft loss [3]. Inflammation, fibrosis and accelerated arteriosclerosis are typical features for chronic allograft injury.

Vascular smooth muscle cells (VSMC) and monocyte–macrophages play a central role in the pathogenesis of chronic allograft injury [4,5]. Proliferation and abnormal accumulation of VSMC are among the key events in the development of various vascular diseases, including arteriosclerosis and post-angioplasty restenosis. In chronic allograft injury accelerated form of classical arteriosclerosis is seen in occluding arteries of degenerating grafts [4]. Monocyte–macrophage accumulation has also a critical role during the progression of accelerated vascular disease associated with chronic inflammation [6]. During the development of chronic allograft injury the proliferation and migration of VSMCs as well as monocyte–macrophage proliferation and differentiation involve a complex regulation of numerous cytokines and growth factors.

The epidermal growth factor receptor (EGFR) family and its ligands serve as a switchboard for the regulation of multiple cellular processes. Its function and role in many pathological responses are only beginning to be elucidated while it has been clear that EGFR activity is essential for normal development since its discovery in the mid 1970s. EGFR is the inaugural member of the ErbB family of receptor tyrosine kinases and consists of an extracellular binding domain, a single α-helical trans-membrane domain, an intracellular tyrosine kinase domain, and a carboxy (C)-terminal region that contains autophosphorylation sites

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[7,8]. Upon ligand binding, EGFR undergoes a transition from an inactive monomer to an active homodimer or heterodimer with other four ErbB family members. EGFR dimerization stimulates its intracellular protein kinase activity. In addition to the classical pathway of action, EGFR can be activated through G protein-coupled receptors (GPCRs) without direct interaction with GPCR agonists, an event referred to as transactivation [9]. For example TGF-β, angiotensin II, endothelin 1, aldosterone and thrombin are known EGFR transactivation inducers [10–14]. Thus, it is possible that the signaling stimulated by diverse stimuli would converge on EGFR. All four members of the EGFR family of receptors are expressed on VSMCs, and EGFR is expressed by monocytes and macrophages [15].

Recently we have demonstrated that EGF inhibition by erlotinib inhibits neointimal proliferation in aorta denudation model mimicking transplant vasculopathy [16]. Erlotinib also ameliorates the development of chronic allograft injury in experimental kidney transplantation [16]. Erlotinib is a novel oral highly selective tyrosine kinase inhibitor for EGFR [17]. Its concentration required to inhibit PDGF tyrosine kinase is over 1000-fold of that of EGF tyrosine kinase inhibition [17]. Erlotinib is clinically used for non-small cell lung cancer (NSCLC) and pancreatic cancer and it prolongs progression free survival in both of these malignancies [18,19]. According to clinical studies erlotinib is well tolerated [18]. To further study the cellular effects of EGFR on chronic allograft injury we studied here the role of erlotinib in VSMC and monocyte–macrophage cell cultures in vitro.

2. Material and methods

2.1. VSMC cell culture

Rat coronary artery SMCs (kindly provided by Professor Dariusz Leszczynski; Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland) were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS), 1% glutamine (Invitrogen), 2.5% penicillin and streptomycin (Invitrogen). The cells were grown at 37 °C in humidified atmosphere with 5% CO_2. Cell growth was monitored by microscoping the cells by an inverted microscope.

2.1.1. VSMC proliferation studies

For the proliferation study, 500 cells were cultured in 96-well culture plates for 24 h following serum starvation in serum free media for 72 h. After serum starvation, the cells were stimulated to proliferate with 2 ng/ml of rat recombinant PDGF-BB (Sigma-Aldrich, St. Louis, MO), a known VSMC inductor [20,21]. Unstimulated cells were used as the control group to reveal stimulation effect. After the stimulation, the cells were treated with erlotinib (1, 2 or 5 nM) or left untreated. Erlotinib was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). After a 24-h stimulation, cell proliferation was determined by using a MTS Assay (Celltiter 96, Promega) and also by visual cell count. Ten microliters of MTS/PMS (phenazine methosulfate) solution was added to the wells for 4 h, and light absorbance at 540 nm was determined with an enzyme-linked immunoadsorbent assay plate reader (Benchmark Microplate Reader, Bio-Rad Laboratories, Hercules, CA). Absorbance from wells with no cells was reduced from the results. The experiment was repeated three times and was done in triplicates. Cell proliferation index was calculated according to the proliferation results, index number 1 represents control cells.

2.1.2. VSMC migration studies

VSMC migration was quantitated in Transwell culture chambers (Corning Inc., Corning, NY) where the upper and lower culture chambers are separated by a polycarbonate filter with 8-μm pores. The chambers were first coated with collagen (Rat Tail Collagen, Type 1, Sigma-Aldrich) at a concentration of 20 μg/ml at +4 °C for 24 h. After 72 h serum starvation rat coronary artery VSMCs were seeded in the upper chamber. PDGF-B was added to the lower chamber in DMEM supplemented with 0.5% FCS and 0.1% BSA. The cells were treated with erlotinib (1, 2 or 5 nM) dissolved in DMSO or left untreated. After 24 h, the filters were removed, fixed with methanol, and stained with Mayer's hematoxylin and eosin. Migrated cells (on the lower side of the filter) were quantitated by counting specified cross-sectional fields with a light microscope using 400× magnification. The experiment was repeated three times and was done in triplicates. Cell migration index was calculated according to the migration results, index number 1 represents control cells.

2.2. Monocyte–macrophage cell culture studies

As a model for monocyte–macrophage proliferation and differentiation human monocytic cell line U937 [22–24] were used. The U937 cells (kindly provided by Dr K. Nilsson, University of Uppsala, Sweden) were kept at logarithmic growth in RPMI-1640 (Lonza) containing 10% fetal bovine serum (FBS), 1% glutamine (Invitrogen), 2.5% penicillin and streptomycin (Invitrogen). The cells were grown at 37 °C in humidified atmosphere with 5% CO_2. Cell growth was monitored by microscoping the cells by an inverted microscope and counting the cells in a cell chamber. Viability of the cells was determined by Trypan blue exclusion.

2.2.1. Monocyte–macrophage proliferation studies

[^{3}H]thymidine incorporation studies were performed to test the effect of erlotinib on monocyte–macrophage proliferation. The cells were treated with erlotinib (1, 2 or 5 nM) or left untreated. Erlotinib was dissolved in DMSO. After 1 day culture, for the measurement of DNA synthesis, 100 μCi/ml of [^{3}H]thymidine (20 Ci/mmol, PerkinElmer) was added to each well for the last 4 h of culture. The cells were then harvested and transferred on paper filters using a cell harvester (Skatron Combi, Skatron, Norway) and ^{3}H-radioactivity was measured in liquid scintillation counter. The results are presented as mean ± SE from three individual experiments done with three parallels.

2.2.2. Monocyte–macrophage differentiation studies

10^{-8} M phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Sigma-Aldrich) was used to induce the cells to differentiate into macrophages. Morphology of the TPA-treated U937 cells was analyzed after 1, 2 and 3 days' exposure to erlotinib (1, 2 or 5 nM). The cells were cytocentrifuged on cytopreps. Slides were fixed and stained with May-Grunwald-Giemsa stain, and cells were examined under a microscope at 40× magnification to determine the morphologic features of monocyte–macrophage differentiation. The percentage of differentiated macrophages was counted each timepoint. Each experiment was performed in triplicate.

2.3. Statistical analysis

The results are expressed as mean ± SE, and $p < 0.05$ was accepted as significant. The significance between the groups was determined by parametric analysis of variance and least significant difference test, ANOVA or by non-parametric analysis of Mann–Whitney (SPSS version 10; SPSS, Chicago, IL).

3. Results

3.1. Erlotinib in VSMC proliferation

The results of VSMC proliferation experiments with erlotinib are presented in Fig. 1A. PDGF-B, a known VSMC inducer, was used to stimulate cultured VSMCs to proliferate. PDGF-B caused a 2.1-fold stimulation in VSMC proliferation compared with non-stimulated control VSMCs. Erlotinib completely prevented this PDGF-induced VSMC proliferation with all studied doses. In these erlotinib-treated groups proliferation from 0.8 to 0.5 was seen, $p < 0.001$ in all erlotinib groups compared to the untreated PDGF-induced control group. No marked difference was seen between the different erlotinib doses in VSMC proliferation.

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