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Epidermal Dipeptide: A New Regulatory Factor in Proliferative Vitreoretinopathy

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ABSTRACT

Proliferative vitreoretinopathy (PVR) is the most severe complication of retinal detachment surgery, occuring approximately in one out of ten operated eyes. The proliferation of retinal pigment epithelial (RPE) cells and fibroblasts is a cornerstone in the pathogenesis of PVR. Since inhibitory peptides may take a part in the feedback mechanism underlying this proliferation, we assesed the effect of synthesized epidermal dipeptide, pyroglu-glyOH (EDP), on the proliferation of RPE cells and fibroblasts in vitro. The maximum inhibitory effect of EDP on the RPE cells was reached at concentrations ranging from $1.07*10^{-13}$ to $1.07*10^{-15}$ M. Its inhibitory effect on fibroblasts followed a similar pattern at all concentrations applied, $1.07*10^{-6}$ to $1.07*10^{-15}$ M. These results enhance the possibility that PVR may be due to an imbalance of inhibition/disinhibition mechanism under participation of several regulatory molecules like EDP. EDP might have the potential for reducing the risk of PVR.

INTRODUCTION

Proliferative vitreoretinopathy (PVR) leading to traction retinal detachment, is a major cause of blindness in a variety of clinical conditions, including long-standing rhegmatogeneous retinal detachment, and is often a complication of retinal detachment surgery (1,2,3,4,5). In PVR retinal pigment epithelium (RPE) cells, fibroblasts and macrophages can gain access to the vitreous cavity where they adhere to the retina and vitreous, proliferate, acquire myofibroblast-like morphology, synthesize extracellular matrix and eventually contract. This contraction leads to retinal detachment and challenges the successful detachment surgery(6).

In vitro experiments suggest that the proliferation of RPE cells and fibroblasts can be modulated by several growth factors, e.g., transforming growth factor beta (TGF-beta), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF). This implies the existence of a negative feedback mechanisms controlling the initiation and progression of PVR(5).

In several studies it has been shown that quick-acting, short-lasting negative feedback signals, termed chalones, tend to inhibit the proliferation of different cell lines. Chalones act as tissue-specific but not species-specific endogenous inhibitors(7,8,9).

A synthetic epidermal dipeptide (pyroGlu-GlyOH), with semi-chalone activities, has been shown to inhibit the proliferation and differentiation of mouse epidermal cells in vitro (10).

RPE cell and fibroblast cultures provide a useful in vitro model for analysing the effect of different regulatory factors on their proliferation. In the present study, we report the effects of the synthetic epidermal dipeptide on the regenerating cultures of RPE cells and fibroblasts.

MATERIALS AND METHODS

Cell Culture

RPE cells: RPE cells were cultured from pig eyes provided by the local abattoir. The eyes were processed within 2 hours post mortem in the following way: a circular cut 3 mm from the corneal limbus through sclera, chorioid layer, RPE and neural retina allowed the removal of the whole anterior part of the eye. Once the vitreous had been lifted out of its shell, the neural retina was stripped off mechanically and seperated at the optic nerve. The empty shell was rinsed twice in phosphate buffered saline (PBS), and then filled with 0.5 mM ethylenediaminetetraacetic acid (EDTA) and incubated for 10 min at 37° C. RPE cells were harvested by extensively rinsing the eye cup with minimal essential medium (MEM D-Val, Sigma) and then explanted in 3.5-cm^2 dishes (primary culture). After the first passage, the cells were grown in 75-cm^2 flasks (Nunc) in MEM D-(Val) containing 15% fetal calf serum (FCS, Boehringer, Mannheim), supplemented with an antibiotic/antimycotic (50g/ml gentamycin/ 2.5 g/ml amphoterecin, Squibb Pharma) mixture under 5% CO_2. RPE cells were subcultured weekly. Fourth-passage cells were used for long and short assays of EDP.

Fibroblasts: fibroblasts were cultured from human eyes tenon capsules obtained from the Ophthalmology Department of the Medical University of Cologne. The fibroblasts were explanted in 3.5-cm^2 dishes (primary culture). After the first passage, the cells were grown in 75-cm^2 flasks (Nunc) in DMEM (minimal essential medium, Sigma) F-12 containing 15% fetal calf serum (FCS, Boehringer, Mannheim), supplemented with an antibiotic/antimycotic (50 g/ml gentamycin/2.5 g/ml amphoterecin, Squibb Pharma) mixture under 5% CO_2. Fibroblasts were subcultured weekly. Fourth passage cells were used for the long assay of EDP.

EDP Assay

Confluent cultures were rinsed three times with PBS and then trypsinised by covering the cell layer with 0.1% trypsin (Boehringer, Mannheim) containing 0.5 mM EDTA for 5 min. The cells were then resuspended in 3.5-cm^2 dishes at a density of 50000 cells per dish and grown for 24 hours. The cells were rinsed three times with PBS. For the short time assay (RPE cells only), EDP was added at different concentrations every two hours over a period of 24 hours. For the long time assay (RPE cells and fibroblasts), EDP was added repeatedly (8 times) during the first 48 hours(0 h, 5h, 20 h, 22 h, 24 h, 26 h, 28 h ,30h) followed by two additions each in 2 days intervals, and then followed by one addition after 3 days interval. Cell growth determination was performed immediately after removal of the drug.

Proliferation Determination

Cell growth is determined by the application of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) (Sigma). MTT is a water soluble tetrazolium salt, yielding a yellowish solution when prepared in media lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by

mitocondrial active dehydrogenase enzymes. This water insoluble formazan can be solubilized using isopropanol and the dissolved material is measured spectrophotometrically giving absorbance as a function of concentration of converted dye.

In our experiment 100 mg MTT was dissolved in 50 ml PBS, and 150 ml of culture medium was subsequently added. In the short time assay, after 2 hours incubation and in the long time assay at different intervals of incubation with EDP, it was substituted with 100µl of MTT solution, being incubated for 3 hours. MTT was aspirated through a pipet and 100 µl dimethylsulfoxid (DMSO) was added. The results were read spectrophotometrically at a wavelength of 550 nm.

All the values in the graphs are means of two counts of four assays.

RESULTS

RPE Cells

The modulatory effect of EDP on RPE cells was evaluated in a short time and in a long time assay. In the short time assay different concentrations of EDP were added every second hour over a period of twenty four hours and the cell numbers were counted five hours after each addition (MTT-Elisa). The results are shown in figures 1 a, b. In comparison to the control cultures, EDP had a marked inhibitory effect on the proliferation of RPE cells . The maximum inhibitory effect was reached in lower concentrations, i.e. 10^{-11} - 10^{-13} M. After repetitive additions, higher concentrations also tend to suppress the proliferation but the effect is shorter and weaker.

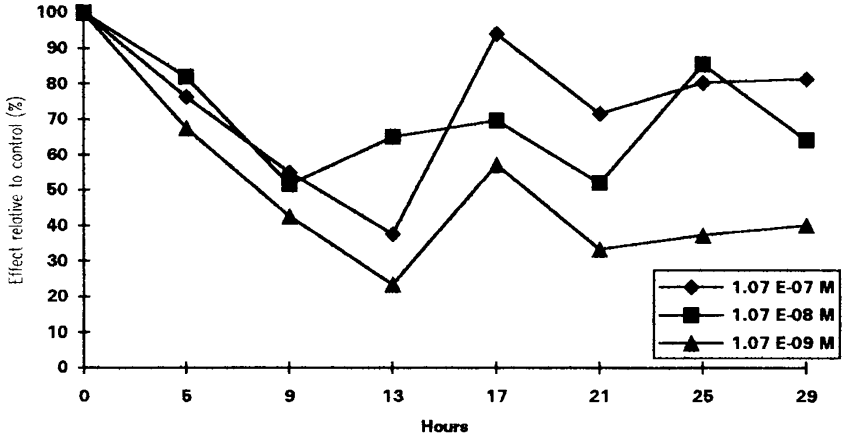

FIG.1 A : Concentration - response diagram showing RPE cells in cultures incubated with various concentrations of epidermal dipeptide administered repeatedly in every second hour over a period of 24 hours.

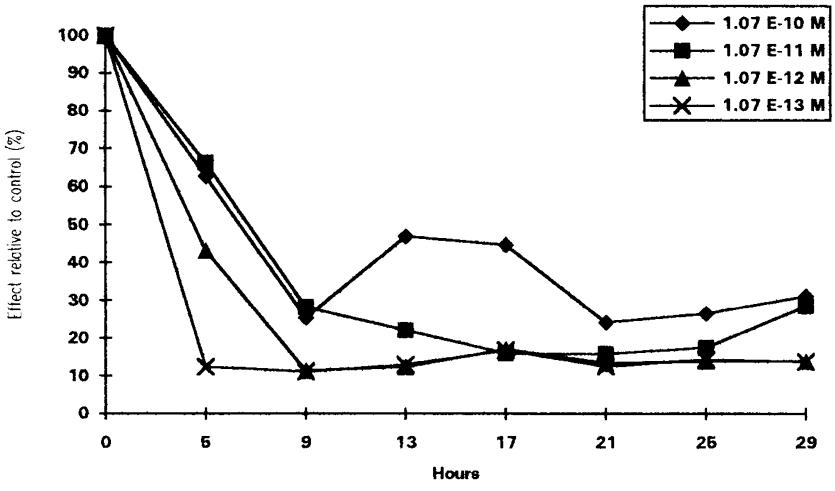

FIG.1 B : Concentration - response diagram showing RPE cells in cultures incubated with various concentrations of epidermal dipeptide administered repeatedly in every second hour over a period of 24 hours.

In the long time assay, the modulatory effect was followed up over a period of 11 days.The EDP was added at different intervals. The results are shown in figure 2. The maximal inhibitory effect was reached in a dose of 10^{-15} M. The suppression tended to be reversible, lasting for 4 to 11 days depending on the concentration.

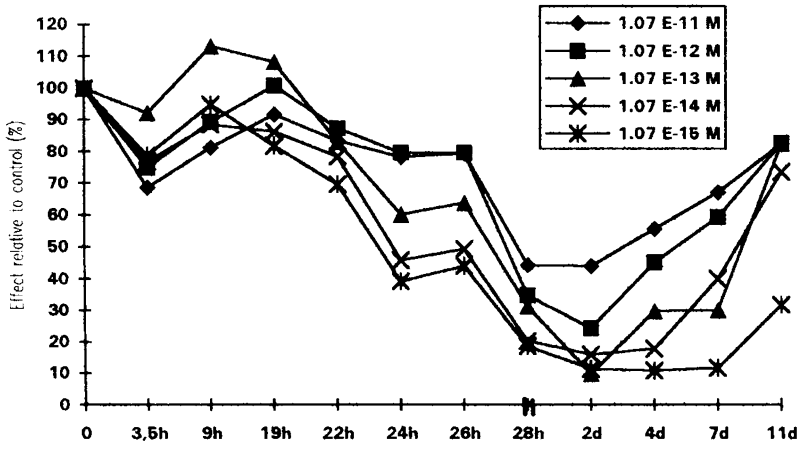

FIG.2 : Concentration - response diagram showing RPE cells in cultures incubated with various concentrations of epidermal dipeptide administered repeatedly over a period of 11 days: 8 times over the first 48 hours(0 h, 5 h, 20 h, 22 h, 24 h, 26 h, 28 h, 30 h), followed by two additions each in 2 days intervals and then followed by one addition after 3 days interval.

Fibroblasts

The effect of EDP on the proliferation of fibroblasts is shown in Figure 3. Although lower concentrations tend to be slightly stronger, all the concentrations showed an increasingly inhibitory effect. The suppression tended to be irreversible, lasting for 11 days.

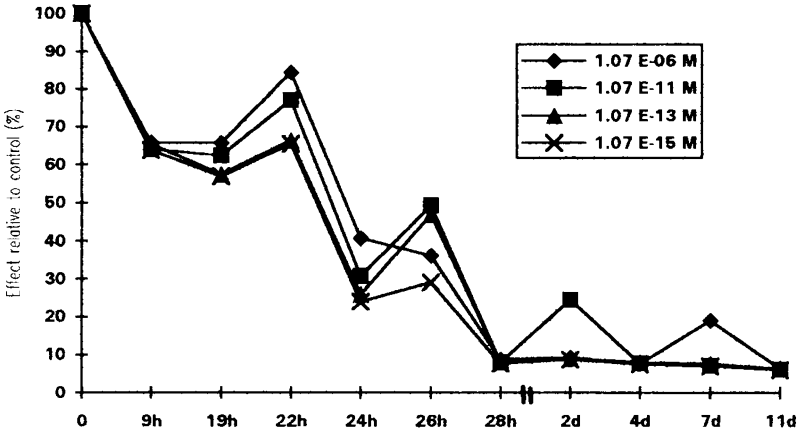

FIG.3 : Human fibroblasts are incubated with various concentrations of epidermal dipeptide administered repeatedly over a period of 11 days: 8 times over the first 48 hours(0 h, 5 h, 20 h, 22 h, 24 h, 26 h, 28 h, 30 h), followed by two additions each in 2 days intervals and then followed by one addition after 3 days interval.

DISCUSSION

Different studies have hypothetised that PVR is an exaggerated wound healing response occuring after intraocular trauma or surgery (1,3,4,5). The evolution and progression of tissue repair can be divided into three phases: inflammatory, proliferative, and remodeling. This model parallels the natural course of PVR (5). It is widely known that the proliferation of RPE cells and fibroblasts are major ethiologic factors in the formation of vitreal and periretinal membranes in PVR (11). Today's model for the control of cell proliferation postulates the existence of both positive and negative signals. Bullough, Lawrence and Iversen developed the chalone theory, which postulates a simple feedback mechanism: proliferating tissues produce tissue specific compounds (chalones), which inhibit the proliferation in the producing cells (7,12). The chalone theory is currently being rejuvenated within the framework of the"autocrine hypothesis" (13). Several regulatory factors have been found to modulate the proliferation of cells in PVR, mostly stimulating it (5). Hence, one of the reasons for the "overhealing" process in PVR may be due to the lack of inhibitory factors, causing the disregulation of the inhibiting/disinhibiting mechanisms controling the healing process in the eye.

The inhibitory effect of the synthetic epidermal dipeptide was originally tested on human epidermis:it inhibited the proliferation and differentiation of human epidermal keratinocytes in vitro. It has been suggested that EDP plays a role in the complex inhibitory/disinhibitory

mechanism of keratinocyte proliferation (10). Parallel to this hypothesis, PVR may also be under control of inhibitory/disinhibitory mechanisms being affected by EDP.

In this study, we report for the first time, the inhibitory effect of EDP on RPE cells and fibroblasts. The inhibitory effect on the proliferation of RPE cells tended to be reversible, having its maximal effect at concentrations ranging from 10^{-13} to 10^{-15} M. The inhibitory effect declined after 4 to 11 days depending on the concentration. This loss of inhibition may be due to the loss of the cells sensitivity to EDP or due to the reduction of number of intervals (after the first 48 hours) in which EDP was added. Higher concentrations exert a weaker inhibitory effect declining at 10^{-6} M. The reason for this pattern is still not known but this may also be due to the insensitivity of the cells to the higher concentrations of EDP. Further studies are needed to findout EDP's mean of action on the RPE cells. This may also clarify the reason for cell insensitivity at higher concentrations or longer periods of EDP application.

EDP is not tissue specific and also inhibits the proliferation of human fibroblasts. The inhibitory effect had the same strength and pattern in all the concentrations, 10^{-6} to 10^{-15} M. The effect on the fibroblasts, which was opposite to that of the RPE cells, was not refractory and no proliferation increase appeared during the observation time. This suggests that either the inhibition is irreversible or the refraction tends to occur after 11 days.

It has been shown that EDP reduces the proportion of strongly labelled (rapidly cycling) S-phase keratinocytes; there is evidence that the strongly labelled S-phase cells constitute a distinct population responsible for rapid expansion of cell mass both during lateral growth and during regeneration (10,14). EDP may exert the same inhibitory mechanism on RPE cells and fibroblasts as it does on the keratinocytes, but, as in many other regulatory factors, it can also have different roots of action. For answering these questions, as mentioned above, further studies are needed to show the exact pathomechanism of EDP's action and its pharmacokinetiks.

The inhibiting effect of EDP on RPE cells and fibroblasts implies the existence of positive and negative signals modulating PVR and enhances the possibility of its pharmacological use. One may assume that the method of aplication would probably be the intravitreal injection before and during vitrectomy.

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