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Stimulation of human skin fibroblast migration by the neuropeptide secretoneurin

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Abstract

Fibroblasts, besides other cells, are called upon when tissue sustains an immunological, mechanical or chemical injury. Fibroblasts migrate into the site of inflammation, proliferate and synthesize and remodel a new matrix. These cellular responses are mediated locally by the release of neuropeptides from sensory nerve endings. Secretoneurin is a newly discovered 33-amino acid neuropeptide derived from secretogranin II (chromogranin C), which is found in sensory afferent C-fibers. We show here that secretoneurin triggers the selective migration of human skin fibroblasts in vitro, but does not stimulate their proliferation. The attraction of human skin fibroblasts toward secretoneurin could be blocked by specific anti-secretoneurin antibodies and is mediated by the C-terminal fragment of the peptide. The observed activity of this sensory neuropeptide is the first description of a specific effect on human skin fibroblasts and suggests a role for secretoneurin in inflammation and wound healing.

Keywords: Secretoneurin; Fibroblast; Migration; Proliferation

1. Introduction

Sensory nerves mediate the inflammatory reaction to irritating substances in several organs and coordinate the healing process. The peripheral release of neuropeptides by different stimuli not only causes vasodilatation and an increase in smooth muscle tonus at sites of inflammation, but also leads to an interaction with immunocompetent and inflammatory cells including fibroblasts. Thus, substance P, an undecapeptide of the tachykinin family not only causes hyperemia due to vasodilatation, plasma extravasation, and an increase in smooth muscle tonus (Pernow, 1985), but also leads to an interaction with immunocompetent cells (Wiedermann et al., 1989, 1993; Ruff et al., 1985). The recently discovered 33 amino-acid neuropeptide secretoneurin, which is derived from secretogranin II by enzymatic processing is widely distributed throughout the central and peripheral nervous systems (Kirchmair et al., 1993; Marksteiner et al., 1993; Hoflehner et al., 1995). Secretoneurin may play a role in neurogenic inflammation as is suggested by its co-localization with other neuropeptides, such as substance P in afferent C-fibers (Marksteiner et al., 1993; Kirchmair et al., 1994). Its release from afferent nerve endings by capsaicin (Kirchmair et al., 1994) and its chemotactic effects on human monocytes both in vitro and in vivo (Reinisch et al., 1993) further corroborate this hypothesis.

Fibroblast migration from neighboring connective tissue into the site of inflammation plays an important role in inflammation and wound repair. In previous studies the well defined neuropeptide substance P was shown to exert chemoattractant activity on neutrophils (Wiedermann et al., 1989), eosinophils (Wiedermann et al., 1993), monocytes (Wiedermann et al., 1989; Ruff et al., 1985) and on human skin fibroblasts (Kähler et al., 1993a). Given the close proximity of sensory nerves to subepithelial fibroblasts, we hypothesized that secretoneurin might influence fibroblast activity and therefore modulate the subepithelial deposition of connective tissue. We therefore studied whether the neuropeptide secretoneurin could affect human fibroblast proliferation and migration in vitro.

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2. Materials and methods

2.1. Peptide purification and production of antisera

Human secretoneurin was obtained from Neosystem, Strasbourg, France. Two further peptides derived from the primary amino acid sequence of secretogranin II and secretoneurin antibody were kindly prepared by H. Winkler and R. Fischer-Colbrie, Department of Pharmacology, Faculty of Medicine, University of Innsbruck as described previously (Kirchmair et al., 1993): bovine secretogranin II (133–151) and human secretogranin II (carboxy-terminal fragment of secretoneurin). For antisera production secretoneurin was coupled via an additional N-terminal cysteine to maleimide activated keyhole limpet hemocyanin and used for generation of antisera in Chinchilla bastard rabbits (Ivanovas, Kislegg, Germany) as already reported in detail (Kirchmair et al., 1993). Immunoglobulin fractions were prepared from antiserum by affinity chromatography on Protein A Sepharose columns (Pharmacia, Bromma, Sweden).

2.2. Human skin fibroblast culture

Human skin fibroblasts were isolated from two human skin fragments (kindly provided by N. Romani, Department of Dermatology, Faculty of Medicine, University of Innsbruck) and utilized in passages 3 to 8. Fibroblast monolayers were cultured in enriched M199 (Biological Industries, Beth Haemek, Israel) in 175 cm^2 plastic tissue culture flasks and kept in a humidified incubator at 37°C and 5% CO_2. After reaching confluence the cells were subcultured and reseeded at a ratio of 1 : 3.

2.3. Chemotaxis and chemokinesis assays

Chemotaxis of human skin fibroblasts toward the test attractants was measured using a modified Boyden chamber with a 6.5 mm, 8 μm pore size, 10 μm thickness polysterene membrane separating the two chambers (Transwell, Costar, NL) as described previously (Leavesley et al., 1993). The upper surface of the filter was coated with fibronectin at a concentration of 10 μg/ml. Assay substances were dissolved in M199 with 0.5% bovine serum albumin (Behring, Marburg, Germany), placed in the lower compartment of a Transwell and allowed to stand at 37°C for 30 min before placement of the cells in the insert (10^6 cells/ml). Single-cell suspensions of human fibroblasts were prepared from confluent monolayer cultures. Fibroblasts were first washed with Hepes/EDTA and then treated with 0.05% trypsin and 0.02% EDTA at 37°C until the cells were observed to round up. M199 containing 20% fetal calf serum was then added to the cultures to stop trypsinization. Following centrifugation, the cells were resuspended in M199 supplemented with 0.5% bovine serum albumin and allowed to recover for 45 min at 37°C before placement in the upper compartments of the Transwells. Cell migration was measured after 6 h of incubation at 37°C. All non-migrant cells were removed from the upper surface of the Transwell membrane with a cotton swab and migrant cells, those attached to the lower surface, were fixed and stained with Diff Quick (Dade, Düdingen, Switzerland). Stained cells were extracted with 10% acetic acid, absorbance at 600 nm was determined, and migration was calculated from a standard calibration curve. In all experiments, we used epidermal growth factor at a concentration of 10 μg/ml as positive chemotactic control (Grant et al., 1992) and medium supplemented with 0.5% bovine serum albumin as negative control to determine random migration.

Assessment of random migration as opposed to movement along a gradient (chemotaxis) was determined by incorporating the same concentration of chemoattractant into both the upper and the lower compartment of each well (checkerboard analysis).

2.4. Proliferation assay

To assess the effect of secretoneurin on proliferation, fibroblasts were seeded (7.5 × 103 cells/well) onto 96-well microtiter plates in M199 with 0.2% fetal calf serum. After a 48 h preincubation, the medium from each well was replaced with log dilutions of secretoneurin in M199 with 0.5% fetal calf serum. After a further 72 h in culture, fibroblast proliferation was assessed using a colorimetric assay based on the tetrazolium salt [3-(4,5 dimetyldiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] (MTT; Sigma, St. Louis, MO) as described previously (Kähler et al., 1993b; Mosman, 1983; Twentyman and Luscombe, 1987). At the end of the incubation period MTT was added to each well of the microtiter plate. After a further 6 h of incubation the medium was aspirated, dimethylsulfoxide was added to each well to dissolve formazan crystals and the absorbance measured at 630 nm, using a microplate spectrophotometer. Controls (negative/positive) consisted of M199 supplemented with 0.5% or 10% fetal calf serum, respectively.

3. Results

The purpose of this study was to evaluate whether the recently discovered neuropeptide secretoneurin can function as a chemotactic and/or proliferative stimulus for human skin fibroblasts. Thus, we first tested the ability of human skin fibroblasts to migrate in response to the new neuropeptide over a broad concentration range (from 10^{-6} to 10^{-12} M). Our results showed a significant migratory response of human skin fibroblasts in vitro (Fig. 1). The observed migratory effect was concentration-dependent, showing a bell-shaped dose-response curve with a peak response occurring at 10^{-10} M. The peak migratory effect on human skin fibroblasts toward secretoneurin was com-

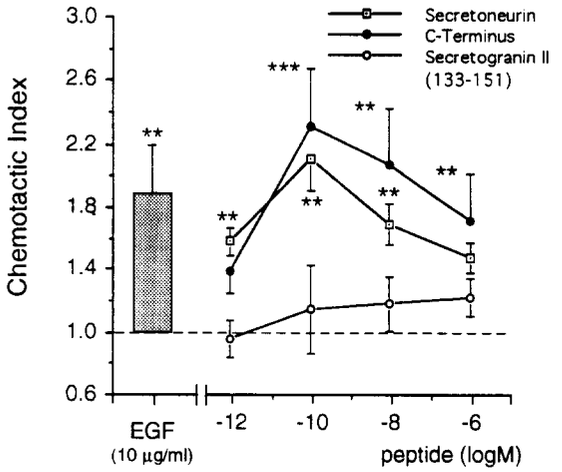

Fig. 1. Human skin fibroblast migration in response to secretoneurin and two other secretogranin II cleavage products (secretoneurin [133–151], C-terminus) in vitro. Dose-response curves are shown at log M dilutions. The number of cells that had migrated to the lower surface of the filters was determined colorimetrically. The results are expressed as the means ± S.E.M. of the chemotactic index ($n = 7$). The concentration of epidermal growth factor was 10 µg/ml and the incubation time was 6 h. The mean optical density of the vehicle control was 0.097 ± 0.025. ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney U-test.

parable to the chemotactic effect seen in the control group treated with epidermal growth factor (10 µg/ml). We furthermore evaluated whether the observed effect is mediated by the C-terminal fragment of secretoneurin. As known from another neuropeptide, substance P, the migratory response of human skin fibroblasts could also be elicited by the C-terminus of the peptide to an extent observed with the whole 33-amino acid peptide (Fig. 1). These results suggest that the C-terminus of secretoneurin is necessary to promote human fibroblast migration. In further experiments we determined whether the observed effect is also mediated by another secretogranin II fragment 133–151, namely LF-19. For this purpose we tested the secretogranin II fragment 133–151 (LF-19) at a concentration range from 10^{-6} to 10^{-12} M on chemotactic activity. As observed in experiments involving human monocytes, this secretogranin II fragment did not elicit a significant migratory response at the concentrations tested.

To further confirm that the observed migratory response of human skin fibroblasts is due to a specific effect of the neuropeptide, we evaluated the cellular response in the presence of specific secretoneurin antibodies. As shown in Table 1, a specific anti-secretoneurin serum at a final dilution of 1 : 1000 was able to abolish the neuropeptide-induced (10^{-10} M) migratory response of human skin fibroblasts.

A checkerboard assay was further performed to determine whether the migratory response of human fibroblasts evoked by secretoneurin represents chemotaxis and/or chemokinesis (Table 2). Secretoneurin elicited movement of human skin fibroblasts against a positive concentration gradient with a maximal chemotactic response between 10^{-8} and 10^{-10} M. Significantly less stimulation of migration was observed toward negative gradients of secretoneurin. Thus, this peptide preferentially stimulates directed migration of fibroblasts in vitro.

Since well known neuropeptides not only induce cellular migration but also often affect their proliferation, another aim of this study was to investigate whether secretoneurin could also modulate fibroblast growth. Human skin fibroblasts seeded at a concentration of 7.5×10^3 cells/well were serum-starved and subsequently stimu-

Table 1
Effect of anti-secretoneurin antibodies on secretoneurin-induced fibroblast migration

	Without anti-secretoneurin antibodies	With anti-secretoneurin antibodies
Vehicle control	1.000 ± 0.000	1.000 ± 0.000
Secretoneurin (10–10 M)	1.587 ± 0.146 [a]	1.101 ± 0.285
Epidermal growth factor (10 µg/ml)	1.879 ± 0.295 [a]	1.631 ± 0.125

Effect of anti-secretoneurin antibodies (1 : 1000) on secretoneurin-induced (10^{-8} M) human fibroblast migration. Human fibroblast migration was examined in the Transwell cell culture chamber as described in Materials and methods. The number of cells that had migrated to the lower surface of the filters was determined colorimetrically. Values represent the means ± S.E.M. of the chemotactic index ($n = 8$). [a] $P < 0.01$, Mann-Whitney U-test.

Table 2
Effect of various concentration gradients of secretoneurin on human fibroblast migration in vitro

Secretoneurin (log M), lower chamber	Migration (CI, mean ± S.E.M., $n = 3$)			
	Secretoneurin (log M), upper chamber			
	0	10–12	10–10	10–8
0	0	0.921 ± 0.125	0.896 ± 0.096	0.975 ± 0.011
10–12	1.401 ± 0.266	1.569 ± 0.144	1.288 ± 0.217	1.333 ± 0.202
10–10	1.703 ± 0.147	1.407 ± 0.088	0.928 ± 0.230	0.763 ± 0.148
10–8	1.919 ± 0.297	1.063 ± 0.311	0.831 ± 0.145	0.957 ± 0.033

Effect of various concentration gradients of secretoneurin on human fibroblast migration in vitro. Values represent the means ± S.E.M. of the chemotactic index after 10 h of incubation. Different concentrations of secretoneurin were added to the upper and/or lower compartment of chemotaxis chambers.

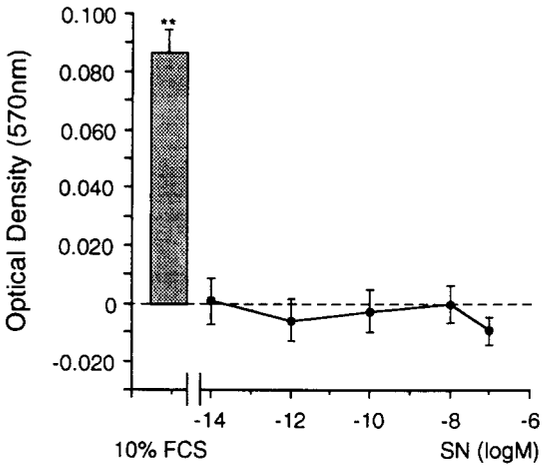

Fig. 2. Effect of secretoneurin on human skin fibroblast proliferation in vitro. Fibroblasts made quiescent in 0.2% fetal calf serum were switched to fresh 0.5% fetal calf serum when log dilutions of secretoneurin were added. The MTT assay was carried out after further 72 h of incubation. Values represent the means ± S.E.M. ($n = 8$) minus optical density of the vehicle control. The mean optical density of the vehicle control was 0.430 ± 0.026. ** $P < 0.01$, Mann-Whitney U-test.

lated with the substances tested. Secretoneurin was tested for its growth-promoting effect over a wide concentration range (from 10^{-7} to 10^{-14} M, Fig. 2). The neuropeptide exhibited no effect on cellular growth as measured colorimetrically. Medium supplemented with 10% fetal calf serum (control group) significantly stimulated human skin fibroblast growth when compared with the effect of vehicle.

4. Discussion

Besides neutrophils and macrophages, fibroblasts are the main cellular component of inflammatory responses. Their migration from neighboring connective tissue into the site of inflammation and their proliferation is a key event in inflammatory responses (Payan, 1992). Their function at inflammatory sites is modulated by distinct molecular signals, including those provided by cytokines and complement. Furthermore, neuropeptides have been implicated in the modulation of a variety of cellular responses in neurogenic inflammation and wound healing. Thus, substance P, for example, plays a major role in regulating cellular proliferation and migration (Payan, 1992; Skerret, 1990; Pernow, 1985). Thus, substance P was shown to stimulate proliferation (Kähler et al., 1993a; Ziche et al., 1990) and directed migration of human skin fibroblasts (Kähler et al., 1993b) in vitro.

Secretoneurin is a recently discovered 33-amino acid neuropeptide (Kirchmair et al., 1993) which is co-localized with several other neuropeptides, such as substance P in afferent C-fibers (Marksteiner et al., 1993). Since this peptide is released by afferent C-fibers in response to capsaicin (Marksteiner et al., 1993) and since secretoneurin affects monocyte functions in vitro and in vivo (Reinisch et al., 1993), it seems to be involved in neurogenic inflammatory processes. Consequently, the purpose of this study was to determine the influence of secretoneurin on the migration and proliferation of human skin fibroblasts. To test the migratory properties we performed a series of Transwell assays. Fig. 1 demonstrates that secretoneurin is a potent chemoattractant for human skin fibroblasts over a wide concentration range. The peak migratory response to secretoneurin was comparable to the peak response in the control group treated with epidermal growth factor (10 μg/ml), which was previously shown to significantly stimulate fibroblast migration (Grant et al., 1992). Additionally, we were interested in the structure-activity relationship of secretoneurin. Stimulation of fibroblast migration was induced by both the complete 33-amino acid peptide and its 15-amino acid C-terminal fragment. Another peptide derived from the precursor secretogranin II, namely secretogranin 133–151 (LF-19), did not exhibit chemoattractant activity. These results are consistent with observations made on human monocyte chemotaxis toward secretoneurin (Reinisch et al., 1993) and appear to be specific. Thus, chemoattraction is mediated only by secretoneurin and its carboxy-terminal fragment.

To further explore whether the observed migratory response is due to a specific effect of secretoneurin, we investigated the effect of the neuropeptide in the presence of a specific anti-secretoneurin serum. For this purpose we tested the effect of specific anti-secretoneurin antibodies on secretoneurin-induced fibroblast migration in vitro. The results show that the migratory response of human fibroblasts toward secretoneurin was abolished in the presence of these specific antibodies, indicating that the observed stimulation is due to a specific effect of the neuropeptide.

Directional movement of fibroblasts toward secretoneurin was studied in a checkerboard analysis with the limitation imposed by the use of polycarbonate filters (Wilkinson, 1988). Secretoneurin elicited movement of human skin fibroblasts against a positive concentration gradient. Significantly less stimulation of migration was observed toward negative gradients of secretoneurin. Thus, secretoneurin preferentially stimulates the cells' directed migration, similar to results obtained from migration experiments with transforming growth factor-β (Postlewaithe et al., 1987), epidermal growth factor (Grant et al., 1992) or substance P (Kähler et al., 1993b).

Secretoneurin did not stimulate proliferation of human skin fibroblasts in vitro. In fibroblasts in culture supplemented with 0.5% fetal calf serum, secretoneurin exhibited no effect on cell growth, observed over a concentration range from 10^{-7} to 10^{-14} M. These results are comparable to results obtained with other neuropeptides such as calcitonin gene-related peptide or vasoactive intestinal peptide.

As the inflammatory response is characterized by migration and proliferation of inflammatory cells, including fibroblasts, our findings may have functional significance with regard to processes in neurogenic inflammation and wound healing. Based on the results of the present study, the chemoattractant potency of secretoneurin may be a major phenomenon in neurogenic inflammation. Last but not least, this is the first description of an effect of secretoneurin on human skin fibroblasts.

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