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ENDOTHELIN-3 MEDIATED PROLIFERATION IN WOUNDED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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An *in vitro* model of endothelial cell injury was used to investigate the role of endothelins and related peptides in endothelial repair. Endothelin-3 (10-100 nM) enhanced wound repair over an 18 h period by promoting proliferation, an effect not inhibited by the specific ET_A receptor antagonist BQ-123 (100 nM) or the mixed ET_A/ET_B antagonist PD142893 (10 μM). Like endothelin-3, the ET_B selective agonists [$Ala^{1,3,11,15}$]endothelin-1 and sarafotoxin S6c were able to enhance wound repair over the same dose range. Neither endothelin-1 nor endothelin-2, however, had any effect on endothelial cell wound healing. Inhibition of cyclo-oxygenase or neutralisation of basic fibroblast growth factor did not inhibit this endothelin-3-mediated event. These results suggest that endothelin-3 might have a direct role in endothelial cell proliferation as a response to injury which is not mediated by either of the currently defined ET_A and ET_B receptors.

The endothelin family of 21 amino acid peptides, and their relatives the sarafotoxins, are best known for their vasoactive properties; the parent peptide endothelin-1 is 10 times more potent as a vasoconstrictor than its nearest rival angiotensin II (1) and endothelin-3 is best known for its vasodilator properties at low concentrations (2,3). However, it is becoming increasingly apparent that the endothelins, and indeed other vasoactive peptides such as angiotensin II, have an important role to play locally in addition to their influence on vascular tone. Indeed, evidence is mounting to suggest that the local actions of endothelins may be likened to those of classical cytokines (4), and much evidence has accrued demonstrating that endothelin levels are in fact modulated by cytokines such as tumour necrosis factor, transforming growth factor-β, interleukin-1 and interferon-γ (5,6). Conversely, endothelins have been shown to modulate the production of a variety of endothelial cell products including other endothelins, prostacyclin and tissue plasminogen activator (7,8) and to promote proliferation of some cell types, particularly smooth muscle (9).

In addition to cytokines, other physiological factors such as shear stress and the presence of thrombin have also been shown to modulate endothelin levels (1,10). Taking observations such as these into account, it has been postulated that elevated levels of endothelins might be anticipated in regions of injury and/or inflammation (11). Therefore, the aim of this study, was to see if exogenous endothelins would influence wound repair in an *in vitro* model of endothelial cell injury.

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MATERIALS AND METHODS

Cell culture: Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins by collagenase digestion not more than 48 h after delivery using the method by Jaffe *et al.* (12). Cells were grown to confluence by incubation at 37°C in a 5% CO_2 atmosphere in 25 cm^2 flasks in complete medium consisting of Medium E199 (ICN Flow Laboratories, Irvine, Scotland) supplemented with 15% fetal calf serum (Advanced Protein Products, Brockmoor, West Midlands, England), L-glutamine (2 mM), penicillin (50 i.u./ml) and streptomycin (50 µg/ml; all from ICN Flow Laboratories). Flasks confluent within 7 days were trypsinised and cells seeded onto 13 mm diameter Thermanox plastic coverslips (Nunc Inc, Naperville, IL, U.S.A.) coated with 0.2% gelatin in 24-well plates. Cells were allowed to attain confluence (2-3 days) and used in the wounding assay described. All plasticware was from Greiner Labortechnik (Gloucester, England) and Trypsin EDTA and Ca^{2+}/Mg^{2+} free PBS were obtained from ICN Flow Laboratories.

Wounding and data processing: A series of parallel wounds was made on each confluent coverslip using a Perspex wounding comb (13). The coverslips were rinsed and placed in a new well containing 450µl complete medium and 50µl drug or vehicle (medium E199). The wounded coverslips were then returned to the incubator for 18 h. The number of cells per coverslip was determined by trypsinisation and the cells counted by the method of trypan blue exclusion.

For the determination of migration, cells were fixed *in situ* at 18 h post-wounding by washing the coverslips 3 times in Ca^{2+}/Mg^{2+} free PBS, and then adding 500µl absolute alcohol to the wells for 5 min. Wells were then aspirated and allowed to air dry. The redistribution of HUVEC into the denuded region of the coverslips was quantified using a VIDS III image analysis system (AMS, Cambridge, England) coupled to a Nikon Diaphot microscope via a Kestrel 25 TV camera. A mean area of wound remaining was taken from 2 fields of view per coverslip, and the percent recovery for each coverslip calculated as [100 x (1 - Final wound area/Initial wound area)]

Drug treated and control wells were run at least in quadruplicate and each experiment was repeated a minimum of 3 times. Statistical significance was determined by analysis of variance. Significance was defined as $P < 0.05$.

Peptides and drugs: Endothelin-3 was from Peninsula Laboratories (Europe) Ltd (St Helens, England); endothelin-1 and sarafotoxin S6c from The Peptide Institute (Osaka, Japan); [$Ala^{1,3,11,15}$]endothelin-1 and BQ-123 (CYCLO[-D-Trp-D-Asp-Pro-D-Val-Leu]) from Neosystem Laboratoire (Strasbourg, France). Actinomycin D was obtained from Novabiochem-Calbiochem (Nottingham, U.K.), indomethacin and gelatin from Sigma Chemical Co. (Poole, Dorset, England). PD142893 (Ac-[βPhenyl]-D-Phe-Leu-Asp-Ile-Ile-Trp) was a gift from Dr A. Doherty, Parke-Davis Pharmaceutical Research (Ann Arbor, MI, U.S.A.). Basic fibroblast growth factor (bFGF), was a gift from Dr M. Presta (University of Brescia, Italy) and the bFGF neutralising antibody, DG2, a gift from Dr T.M. Reilly (DuPont Merck Pharmaceutical Co., Wilmington, DE, U.S.A.).

RESULTS

Image analysis did not detect any change in the rate of recovery of wounded HUVEC monolayers over 18 h post-wounding in the presence of either 1-100 nM endothelin-1 (control, 35.1±2.2% recovery; 100 nM endothelin-1, 35.1±1.5%, n = 4) or 1-100 nM endothelin-2 (control, 32.3±2.0%; 100 nM endothelin-2, 32.2±2.1%, n = 3). However both endothelin-3 (Figs. 1 & 2), and the selective ET_B receptor agonists [$Ala^{1,3,11,15}$]endothelin-1 and sarafotoxin S6c, all facilitated the recovery of HUVEC in a concentration-dependent fashion over this period, compared to their individual untreated controls (Fig. 2).

In order to determine if recovery from wounding involved an increase in cell number, HUVEC were harvested and counted at the end of the experiment. Figure 3A shows that endothelin-3 caused a concentration-dependent increase in the number of cells harvested from the coverslips over the 18 h time course of the experiment, compared to untreated controls. Treatment of the cells with actinomycin D (0.1 µg/ml) did not affect control recovery from wounding, however, the facilitated recovery seen in the presence of 100 nM endothelin-3 was completely abolished by treatment with this mitosis inhibitor (Fig. 3B).

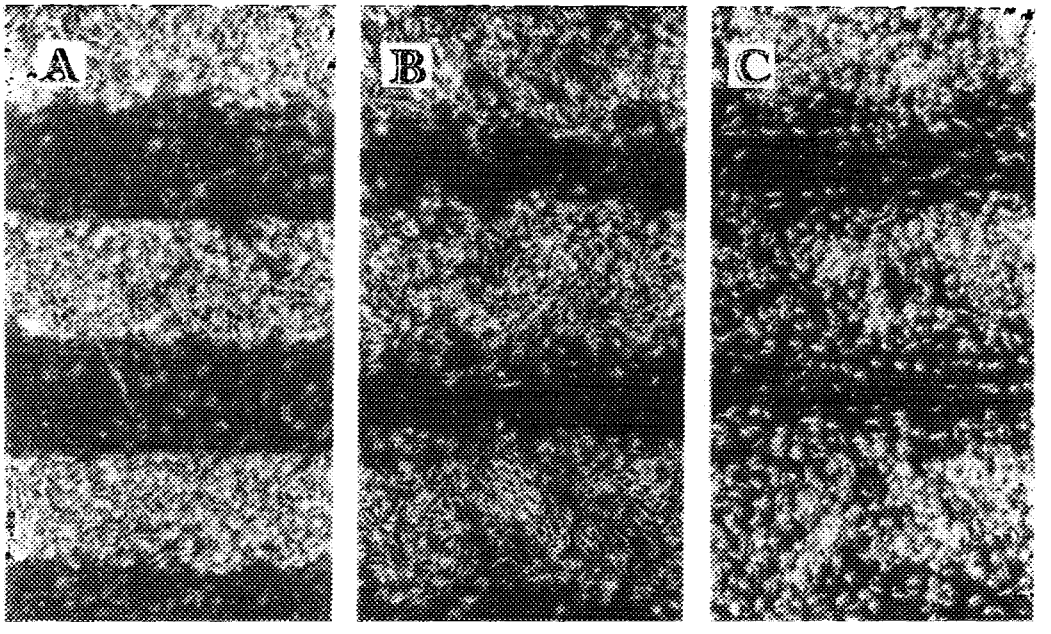

Figure 1. Appearance of cultured HUVEC monolayers: A) immediately post-wounding, B) 18 h post-wounding and C) 18 h post-wounding in the presence of 100 nM endothelin-3.

Two endothelin receptor antagonists were used in order to identify further the subtype of receptor involved in the response of HUVEC to endothelin-3. Figure 4 shows that neither 100 nM BQ-123 (Fig. 4A) nor 10 μM PD142893 (Fig. 4B) antagonised the facilitation of recovery from wounding seen in the presence of 100 nM endothelin-3.

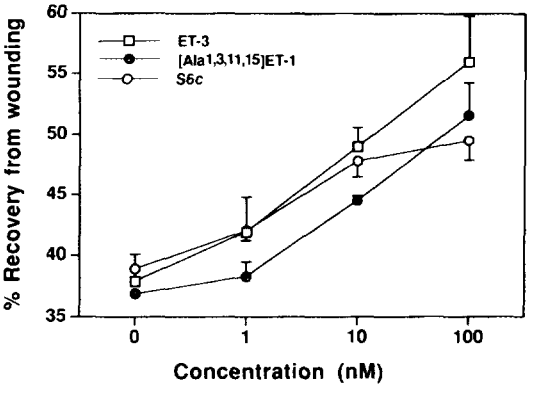

Figure 2. Dose-response relationships for the effects of the agonists endothelin-3 (ET-3; n = 5), sarafotoxin S6c (S6c; n = 5) and [Ala^{1,3,11,15}]endothelin-1 (n = 3) on HUVEC when present for 18 h post-wounding. For all 3 peptides, the recovery at 10 and 100 nM was significantly greater ($P < 0.05$) than the control.

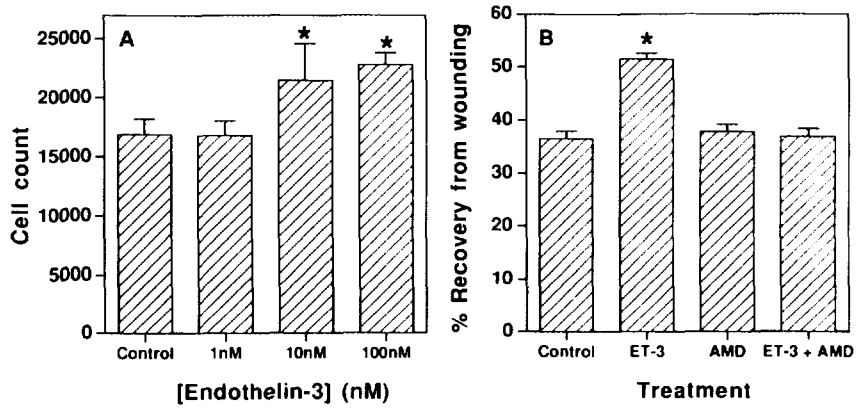

Figure 3. A) Effect of 100 nM endothelin-3 (ET-3) on HUVEC numbers when present in the culture medium for 18 h post-wounding (n = 4). B) The effect of actinomycin D (AMD; 0.1 μg/ml) on the recovery of wounded HUVEC monolayers in the presence and absence of endothelin-3 (100 nM) over 18 h (n = 3). (*) value significantly ($P < 0.05$) greater than the control.

Two possible indirect mechanisms for the actions of endothelin-3 at facilitating the rate of recovery from wounding were investigated by the use of indomethacin (10 μM) and the neutralising bFGF antibody DG2 (100 nM). Although indomethacin significantly inhibited prostacyclin production by the cells (≥ 95% inhibition of prostacyclin levels measured in 2 experiments), Figure 5A shows that it had no effect on either control or endothelin-3-enhanced recovery over 18 h. Similarly, when DG2 was used in the presence or absence of endothelin-3 it had no effect on the response to 100 nM endothelin-3, even though it abolished the increased rate of recovery due to 10 ng/ml bFGF (Fig. 5B).

DISCUSSION

The results show that neither the parent peptide, endothelin-1, nor endothelin-2, which differs from endothelin-1 in only 2 amino acid residues and is the most closely related to the parent of all

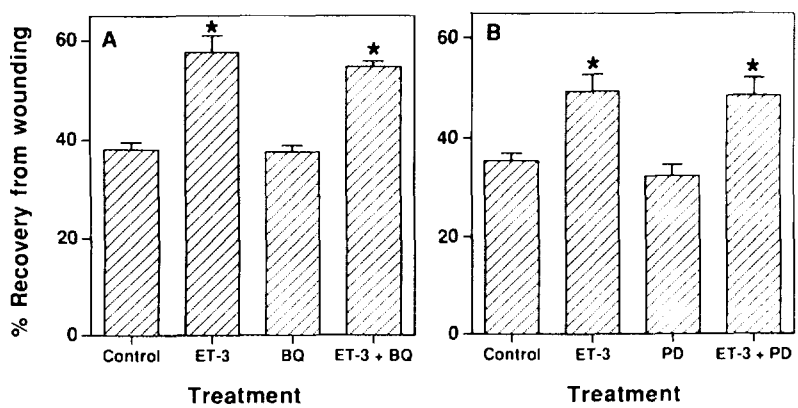

Figure 4. Effect of (A) 100 nM BQ-123, an ET_A selective receptor antagonist (BQ) and (B) 10 μM PD 142893, a non-selective ET_A/ET_B receptor antagonist (PD) on recovery of wounded monolayers of HUVEC in the presence and absence of 100 nM endothelin-3 (ET-3). (*) significantly different ($P < 0.05$) from the control values and n = 5 in all cases.

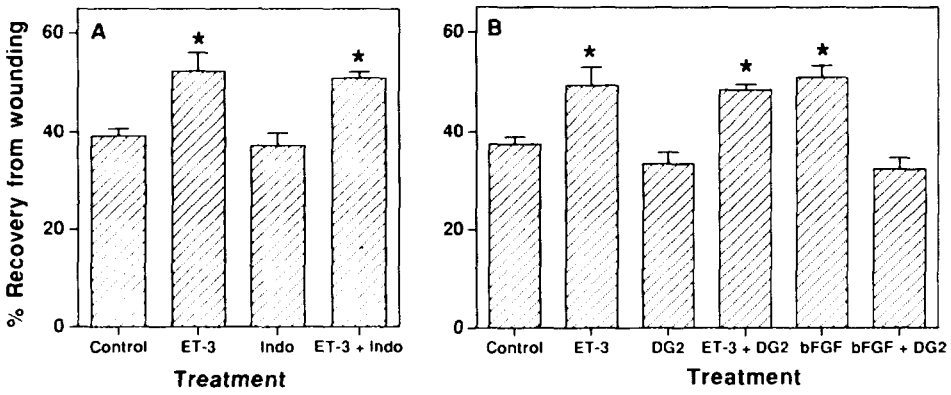

Figure 5. A) Effect of 10 μM indomethacin (Indo) on recovery of wounded HUVEC in the presence and absence of 100 nM endothelin-3 (ET-3) over 18 h (n = 4). B) Effect of the anti-bFGF antibody, DG2, (100 ng/ml) on wounded monolayer recovery in the presence and absence of 100 nM ET-3 and bFGF (10 ng/ml). The results are from 3-5 experiments. (*) shows the values are significantly different ($P < 0.05$) from the control.

the endothelin/sarafotoxin peptides, had any effect on the rate of recovery from wounding displayed by HUVEC monolayers. In contrast, endothelin-3, sarafotoxin S6c and [$Ala^{1,3,11,15}$]endothelin-1 caused significant and concentration-related increases in the rate of recovery from wounding over the 18 h immediately following inflicting the wound.

At present, two types of receptor for the endothelins have been cloned and sequenced (14,15). The ET_A receptor is selective for endothelin-1 with a rank order of potency endothelin-1 > endothelin-2 >> endothelin-3, and is found in the vasculature exclusively on smooth muscle cells where it is responsible for characteristically long lasting vasoconstriction and cellular proliferation (1,9). Inhibition studies using radiolabelled ligands have shown that ET_B receptors however, do not distinguish between the three isopeptides and, here, endothelin-1 and endothelin-3 both display similar affinities for this receptor (15). Both sarafotoxin S6c and [$Ala^{1,3,11,15}$]endothelin-1 have been proposed to be selective agonists at the ET_B receptor subtype (16,17) and so their activity at enhancing wound recovery in HUVEC might, at first, be taken to suggest that the responses observed in the present study are mediated by this receptor subtype. However, endothelin-1 and endothelin-2 were inactive in these experiments which suggests that the enhanced recovery rate was not due to the previously described ET_B receptor.

This conclusion is supported by the observations that neither BQ-123, a cyclic pentapeptide which is a potent antagonist at the ET_A receptor (18), nor the mixed ET_A/ET_B receptor antagonist PD142893 (19) inhibited the response to endothelin-3. Thus, a receptor selective for endothelin-3 amongst the endothelin isopeptides is indicated as mediating the healing response and such a receptor has been reported previously in cultured bovine carotid artery endothelial cells (20) and intact bovine aorta (21). Indeed, activation of an endothelin-3 selective receptor has been reported to induce endothelin-1 production and proliferation in HUVEC (7). Thus the present study confirms that HUVEC possess a receptor selective for endothelin-3. This receptor is not sensitive to either of the endothelin receptor antagonists investigated and its activation can facilitate the

repair processes in an endothelial cell monolayer. Unfortunately, in the absence of a selective antagonist for this receptor, it was not possible to determine whether or not [$Ala^{1,3,11,15}$]endothelin-1 and sarafotoxin S6c were acting on the same receptor to increase the rate of wound healing.

The image analysis system cannot distinguish between recovery due to migration, proliferation, or a combination of the two, but the harvesting of cells followed by cell counting showed that endothelin-3 increased cell numbers over the 18 h after wounding the monolayer. Normally with this model, as in the wounding model described by Sholley and coworkers (22), proliferation of the wounded monolayer is not evident until after 24 h post-injury in untreated wells. The results with actinomycin D show that cell migration is not dependent on *de novo* synthesis of protein, since the drug did not inhibit control rates of recovery. On the other hand, the enhanced recovery seen in the presence of endothelin-3 was completely inhibited and so was due only to an increase in cell number, and not to any increase in the rate of migration.

The known actions of endothelin-3 on endothelial cells include the ability to modulate prostanoid production, in particular prostacyclin (23), and it has been shown that some endothelial cell mitogens, *e.g.* epidermal growth factor, act indirectly to induce proliferation and angiogenesis via prostanoid release (24). The experiments conducted in the presence of 10 μM indomethacin, with and without endothelin-3, showed that this peptide was not acting indirectly through a prostanoid pathway since the cyclo-oxygenase inhibitor had no effect. Similarly, liberation of bFGF from the cellular matrix by endothelin-3 (25) was also investigated as a possible mechanism of indirect action but the neutralising antibody used did not affect the rate of recovery from wounding in the presence of endothelin-3 even though it was able to block the actions of exogenous bFGF.

The present results show that not only endothelin-3, but also other ET_B receptor agonists can cause an increase in the rate of recovery of endothelial cell monolayers from wounding. This effect could not be blocked by the non-selective ET_A/ET_B receptor antagonist, PD142893 and so it would seem that the receptor involved is not identical with other receptors of the ET_B subtype at which these agonists act. If endothelin-3 has a physiological role in wound healing, it would be interesting to know its source. It is generally believed that endothelial cells do not produce this isopeptide (26) but since the present study has used HUVEC it is noteworthy that endothelin-3 mRNA is expressed in human placenta (27). Also, preproendothelin-3-like immunoreactivity has recently been found in a single sample of endometrial endothelium (28). However, since most studies have been carried out in normal vessels, it is possible that endothelin-3 might be expressed only after damage has occurred to a blood vessel. Another local source would be the underlying vascular smooth muscle cells, although again expression of endothelin-3 is not generally reported from this cell type. Although circulating levels of the endothelins have been usually considered too low (of the order of 0.1-10 pM) for them to act as circulating hormones, this has been challenged (29) but it is noteworthy that levels of endothelin-3 often remain unchanged even in pathological conditions in which the levels of endothelin-1 are raised, such as pulmonary hypertension (30). Thus, both the source and the role of endogenous endothelin-3 in repair of vascular injury await further investigation.

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