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IDENTIFICATION OF THE ET_A RECEPTOR SUBTYPE THAT MEDIATES ENDOTHELIN INDUCED AUTOCRINE PROLIFERATION OF NORMAL HUMAN KERATINOCYTES

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SUMMARY: Endothelin-1 has a wide range of pharmacological effects in various tissues and acts as autocrine/paracrine factor. The potential of ET-1 to function as an autocrine growth factor was evaluated in normal human keratinocytes. Radioligand binding studies showed that ^{125}I-ET-1 bound to a single class of high-affinity-binding sites on the surface of the cells. The dissociation constant was 0.045 nM with receptor numbers of 1700 sites/cell. Treatment with serum caused increases in expression of binding sites (3500 sites/cell), with no change in binding affinity. ET-1 stimulated thymidine incorporation in these cells that expressed ET receptors. An ET antagonist selective for the ET_A receptor subtype (BQ 123) inhibited DNA syntesis stimulated by ET-1 and reduced the basal growth rate of unstimulated cells. These data suggest that the ET-1 induced DNA syntesis is mediated by ET_A receptor subtype and that endogenously produced ET-1 promotes the autocrine proliferation of keratinocytes.

Endothelins are a family of three peptides, termed ET-1, ET-2 and ET-3, having profound cardiovascular, mitogenic and neuroregulatory functions. ETs bind to at least two subtypes of G protein-coupled receptors: a selective ET_A receptor that binds ET-1 and ET-2 with high affinity but ET-3 with low affinity and a non selective ET_B receptor that binds all ET isopeptides with equal affinity (1, 2). ET-1 is a potent mitogen for a variety of cell types including vascular smooth muscle

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Abbreviations: ET, Endothelin; NGF, Nerve Growth Factor; bFGF, Fibroblast Growth Factor-basic; TGF-α, Transforming Growth Factor-Alpha; IGF-1, Insulin-like Growth Factor-1; BSA, Bovine Serum Albumin; PBS, Phosphate Buffered Solution; TCA, Trichloroacetic Acid; BPE, Bovine Pituitary Extract; HK, Human Keratinocyte; NHEK, Normal Human Epidermal Keratinocyte.

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cells (3), fibroblasts (4), mesangial cells (5), glial cells (6), melanocytes (7) and tumor cells of epithelial origin (8,9,10). Keratinocytes produce a number of factors influencing the epidermal melanin unit such as NGF (11), bFGF (12), TGFα and IGF-1 (13,14), that stimulate cell proliferation through an autocrine loop. Although it has been found that normal human keratinocytes produce immunoreactive-ET-1 (15,16), it has not been well characterized the role of ET-1 in the keratinocytes growth. In this report, we demonstrate that ET-1 binds ET_A receptors and stimulates growth responses in normal human keratinocytes. These findings, and the inhibitory effect of an ET_A receptor antagonist on cell growth, suggest that endogenously produced ET-1 promotes the autocrine proliferation of keratinocytes.

MATERIALS AND METHODS

Cell culture. Normal human keratinocytes were isolated from foreskins essentially as described by Pirisi et al. (17), except that epidermis was separated from the dermis by overnight digestion with 0.25% trypsin (GIBCO). The keratinocytes were cultivated in a defined medium, keratinocyte serum-free medium (KSFM, GIBCO Inc.) which consists of modified MDCB153 medium with bovine pituitary extract (35-50 mg/ml), calcium chloride (0.09 mM), epidermal growth factor (5ng/ml)(GIBCO Inc.), and antibiotics. Purity of culture was confirmed by cuboidal morphology and positive immunostaining with anti-keratin antibodies. Experiments were performed on cells at second or third passage.

Binding studies. Receptor binding studies with ^{125}I-ET-1 were performed under steady state conditions for 60 minutes at 37°C. Cells were incubated in assay buffer composed of Hanks' balanced salt solution , 0,2% BSA, bacitracin 100 μg/ml, 40 pM ^{125}I-ET-1 and the indicated concentrations of ET related peptides (Peninsula Lab. Belmont, Ca). For equilibrium binding studies, cells were incubated with increasing concentration of the tracer in the presence or absence of an excess (10^{-10} M) of unlabeled ET-1. After 60 min, cells were washed three times with cold phosphate-buffered solution containing 0,2% BSA, then solubilized with 1 M sodium hydroxide and analyzed for bound radioactivity in a γ-spectrometer. The non-specific binding, determined in the presence of 10^{-6} M unlabeled peptide was 3% of the added radioligand. ET-1 receptor affinity and the number of binding sites were determined by Scatchard analysis using the LIGAND program.

Thymidine incorporation assay. Cells were seeded in 96-well plates at approximately 80% confluence and growth factor starved for 24 h to induce quiescence. Selected concentrations of ET-1 and/or competitor BQ 123 were then added and after 18 hours, 1 μCi of [^{3}H]Thymidine (6,7 Ci/mmol, DuPont, NEN) was added to each well. Six hours later the culture medium was removed, the cells were washed three times with PBS, fixed with TCA 10%, washed twice with ethanol 100%, and solubilized with 0,4 N sodium hydroxide. The cell-

associated radioactivity was then determined by liquid scintillation counting. The responses to all agents were assayed in sextuplicate and were expressed as the means of three independent experiments.

RESULTS AND DISCUSSION

In human keratinocytes (HK), specific binding of ^{125}I-ET-1 was a time and temperature-dependent process. Specific binding was rapid, reaching an apparent equilibrium after 60 minutes incubation at 37°C and remained constant up to 90 min (data not shown). ^{125}I-ET-1 binding to HK was saturable (FIG. 1) and Scatchard analysis indicated the presence of a single class of high affinity-sites and a maximum binding capacity of 2.8 fmoles/10^6 cells. The incubation with serum caused on keratinocytes ET-1 binding a 2-fold increase over unprimed cells. Scatchard analysis (FIG.1 inset) of the high affinity binding sites demonstrated that the stimulatory effects of serum were largely accounted by an increase in the number of specific receptors for ET-1. Serum elevated receptor number by 100% over the control value (3852 ET-1 receptors/cell after serum treatment *vs* 1734 in untreated cells). However, receptor binding affinity was not altered by serum as shown by the almost equally dissociation constant for serum treated cells *vs* controls in three different keratinocyte cell lines (TABLE 1). Competition for ^{125}I-ET-1 binding to HK by unlabeled ET-1, ET-2, ET-3 and ET_A-selective antagonist, BQ 123, is shown in FIG.2. Whereas the half-

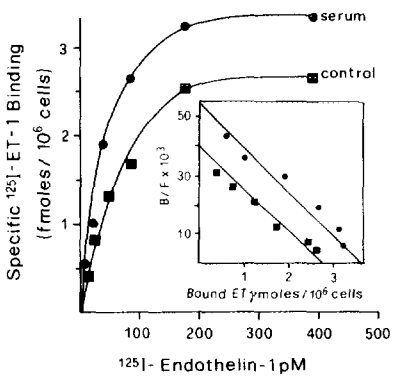
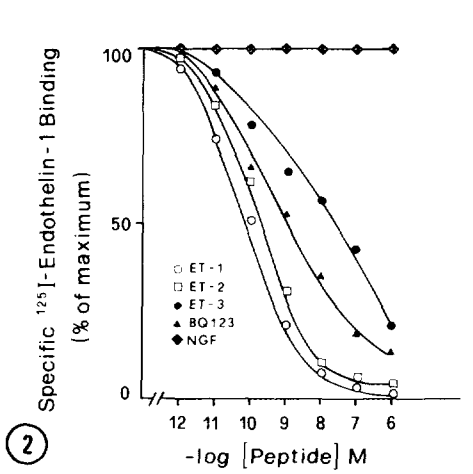


FIG 1. Saturation binding of [^{125}I]-ET-1 to NHEK-1 cell line. Cells were treated in presence or in absence of 10% foetal calf serum for 48 h and then incubated with increasing concentrations of tracer for 60 min at 37°C. Nonspecific binding was determined in the presence of an excess (100 nM) of unlabeled ET-1. Scatchard transformation of the specific binding was calculated and plotted using LIGAND program (inset).

Table I : Binding constants of ET receptors in normal human keratinocyte cell lines

Cell line	receptors/cell		K_d (nM)	
	control	serum	control	serum
NHEK-1	1626	3540	0.042	0.045
NHEK-123	1734	3852	0.045	0.047
NHEK-A56	1831	3132	0.046	0.048



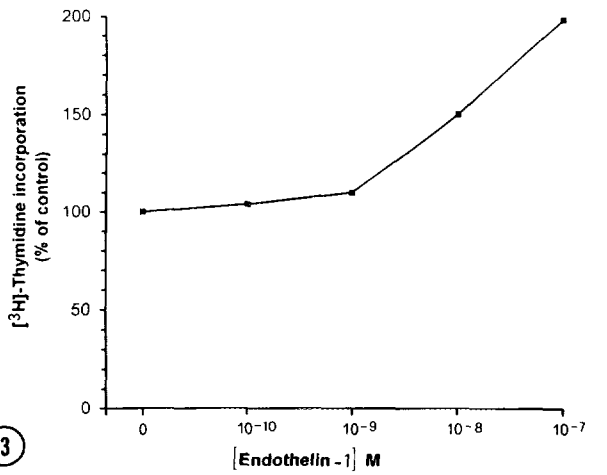


FIG 2. Inhibition of [^{125}I]-ET-1 binding by ET-1 related peptides. NHEK-1 cells were incubated for 60 min at 37°C in the presence of 40 pM [^{125}I]-ET-1 and increasing concentrations of ET-1 related or unrelated peptide. Binding is expressed as a percentage of bound radioligand in the absence of unlabeled peptide . The specificity of binding was indicated by the inability of the unrelated peptide, NGF, to inhibit binding of labeled ET-1.

FIG 3. Effect of increasing concentrations of ET-1 on DNA synthesis by NHEK-1 cell line. Keratinocytes prior to confluence were incubated with ET-1 in growth factor free medium for 18 h and with [^{3}H]Thymidine for 6 h as in methods. Data were means of three independent experiments, each performed in sextuplicate, and were less than 15% of each value ($p<0.05$).

maximum inhibitory concentration (IC_{50}) of ET-1 and ET-2 was $1x10^{-10}$ M and $1.3x10^{-10}$ M, respectively, that of BQ 123 and ET-3 was $1.2x10^{-9}$ M and $5.4x10^{-8}$ M, respectively. In contrast, the unrelated peptide NGF (up to 10^{-6} M) not reduced the specific binding of ^{125}I-ET-1. The differences in potencies of ET-1/ET-2 vs ET-3 in the displacement of labelled ET-1 suggest that the predominant endothelin receptor in these cells is of ET_A subtype. The synthesis and secretion of biologically active ET-1 from basal normal human keratinocytes (16) and the presence of high affinity ET-1 binding sites opened the question about the biological significance of these findings. The role of ET-1 as a growth factor provided a new prospective for considering the biological activity of ET-1 on keratinocyte cultures. As shown in FIG.3 the ET-1 exhibited a dose-dependent increasing effect on thymidine incorporation when the cells were incubated for 24 h in growth factor-free medium. Since ET-1 stimulated mitogenic responses in HK, we evaluated the possibility that ET-1 could act as an autocrine growth factor. The primary requirements for autocrine loop include secretion of a growth-active ligand, the presence of functional cell surface receptors and stimulation of cell proliferation by the ligand. A more rigorous criterion is inhibition of growth by coincubating the cells with a selective receptor antagonist, that specifically blocks the biological action of the growth-promoting ligand. Tsuboi et al. (18), in a recent report, have shown that ET represents a new factor having the capacity to stimulate growth responses in keratinocytes probably through ET_B receptor subtype. They were not able to demonstrate the autocrine role of ET-1 either by inhibiting cell proliferation in the presence of antibody to ET-1 or by using a receptor antagonist. In the present study, addition of ET-1 caused a consistent and dose-dependent increase in DNA synthesis in normal keratinocytes and this stimulatory action was completely blocked by 100 nM BQ 123, an ET_A-selective receptor antagonist, demonstrating that mitogenic signalling occurs through an ET_A receptor subtype (Fig. 4). Furthermore, to determine whether endogenous ET-1 produced by HK exerts a proliferative action, cells were incubated in growth factor-free condition in the absence or presence of 100 nM BQ 123. The inhibition of basal thymidine incorporation by HK in presence of ET_A antagonist, strongly demonstrated that endogenous ET-1 is one of the most important autocrine factors in keratinocyte proliferation. Previous report revealed that ET-1 is a comitogen in that a low concentration of serum or insulin is required for maximal mitogenic activity. In our

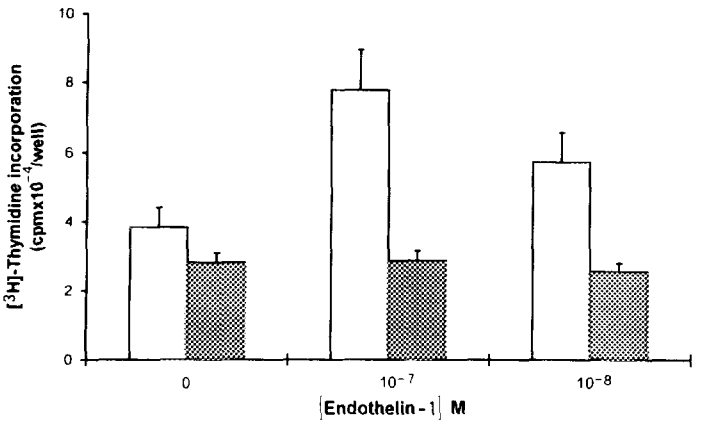


FIG 4. Inhibitory effect of the ET_A receptor antagonist, BQ 123, on ET-1 stimulated incorporation of [^{3}H]Thymidine. The indicated concentrations of ET-1 were added to quiescent NHEK-1 cells that were incubated in growth factor free medium in the absence (open columns) or presence (hatched columns) of 100 nM BQ 123 for 24h prior to[^{3}H]Thymidine incorporation assay. Bars indicated means ± SD of data from three independent experiments, each performed in sextuplicate ($p< 0.01$).

study, we demonstrate that ET-1 induced thymidine incorporation is similar to that induced by BPE, which represent a mixing of several different growth factors, suggesting that ET-1 alone is able to stimulate autocrine growth of keratinocytes as other growth factors. It is possible that ET-1 may exert a mitogenic action on keratinocytes, as well as a paracrine effect on melanocyte growth and melanization (7) or it could participate in the process of angiogenesis as a paracrine growth factor for fibroblasts and endothelial cells. Furthermore our data on serum-dependent increase in ET_A receptor number open the question about an ET-1 involvement in the differentiation pathway, since serum can induce differentiation of primary human keratinocytes. Further studies are in progress to clarify the role of ET-1 in the pathologies involving abnormal keratinocyte proliferation.

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REFERENCES

1- Yanagisawa M., Kurihara H., Kimura S., Tomobe Y., Kobayashi M., Mitsui Y., Yazaki Y., Goto K., and Masaki T. (1988) Nature 332,411-415

2- Sakurai T., Yanagisawa M., and Masaki T. (1992) Trends Pharmacol. 13,103-107
3- Hirata Y., Yoshimi H., Takata S., Watanabe T.X., Kumagai S., Nakajima N., and Sakakibara S. (1988) Biochem. Biophys. Res. Commun. 154,868-875
4- Takuwa N., Takuwa Y., Yanagisawa M., Yamashita K., and Masaki T. (1989) J. Biol. C hem. 264,7856-7861
5- Badr K.F., Murray J.J., Breyer M.D., Takahashi K., Inagami T., and Harris R.C. (1989) J. Clin. Invest. 83,336-342
6- MacCumber M.W., Ross C.A., and Snyder S.H. (1990) Proc. Natl. Acad. Sci. USA 87,2359-2363
7- Yada Y., Higuchi K., and Imokawa G. (1991) J. Biol. Chem. 266, !8352-18357
8- Shichiri M., Hirata Y., Nakajima T., Andok K., Imai T., Yanagisawa M., Masaki T., and Marumo F. (1991) J. Clin. Invest. 87, 1867-1871
9- Kusuhara M., Yamaguchi K., Kuranami M., Suzaki A., Ishikawa S., Moon H., Adachi I., Hori S., and Handa S. (1992) Cancer Res. 52, 3011-3014
10- Bagnato A., Tecce R., Moretti C., Di Castro V., Spergel D.J., and Catt K.J. (1995) Clin. Cancer Res. in press
11- Di Marco E., Marchisio P.C., Bondanza S., Franzi A.T., Cancedda R., and De Luca M. (1991) J. Biol. Chem. 266, 21718-21722
12- Halaban R., Langdon R., Birchall N., Cuono C., Baird A., Scott G., Moellmann G., and McGuire J. (1988) J. Cell Biol. 107, 1611-1619
13- Rothe M., and Falanga V., (1989) Arch. Dermatol. 125, 1390-1398
14- Luger T.A., and Schwarz T. (1990) J. Invest. Dermatol. 95, 100-104
15- Imokawa G., Yada Y., Miyagishi M., (1992) J. Biol. Chem. 267, 24675-24680
16- Yohn J.J., Morelli J.G., Walchak S.J., Rundell K.B., Norris D.A., and Zamora M.R. (1993) J. Invest. Dermatol 100, 23-26
17- Pirisi L., Yasumoto S., Feller M., Doniger J., and Di Paolo J. (1987) J. Virol. 61, 1061-1066
18- Tsuboi R., Sato C., Shi C.M., Nakamura T., Sakurai T., and Ogawa H. (1994) J. Cell. Physiol. 159, 213-220