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Short communication

Enhanced endothelin ET_B receptor down-regulation in human tumor cells

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Abstract

The characteristics of specific binding of human [^{125}I]Tyr^{13}-endothelin-(1–21), [^{125}I]-Tyr^{13}-Suc-[Glu^{9},Ala^{11,15}]-endothelin-(8–21), ([^{125}I]IRL-1620) and endothelin ET_A receptor antagonist [^{125}I]Tyr^{3}-(*N*-[(hexahydro-1*H*-azepin-1-yl)carbonyl]-L-Leu]-1Me)-D-Trp ([^{125}I]PD151242) (number of sites and their affinity) and proliferation responses to exogenous endothelin receptor agonists (endothelin-1 and the endothelin ET_B receptor-selective, truncated *N*-acetyl-[Ala^{11,15}]-endothelin-(6–21) analogue BQ3020) were determined in cultured human fibroblasts and in tumorigenic HeLa cells. The cells were pre-incubated with equimolar concentrations of human endothelin-1 or its truncated analogue BQ3020. After pre-incubation (2 h), both peptides induced down-regulation of surface-membrane endothelin-1 receptors. This process was specific for endothelin ET_B receptors and was much more intensive in tumorigenic cells. BQ3020, acting mostly through its C-terminus, induced nearly maximal endothelin ET_B receptor down-regulation in HeLa cells. Staurosporine, a wide spectrum protein kinase inhibitor, significantly reduced, and *N*-[*N*-[*N*-[2,6-dimethyl-1piperidinyl)carbonyl]-4-Me-L-Leu]-1-(methoxycarbonyl)-D-tryptophanyl]-D-norleucine (BQ788), an endothelin ET_B receptor antagonist, attenuated the down-regulation of endothelin receptors induced by endothelin receptor agonists. The down-regulation of endothelin ET_B receptors was prevented by pre-incubation of the cells with the lysosomal enzyme blocker chloroquine. The endothelin-1-induced cell proliferation was attenuated by pre-incubation of the cells with the non-selective endothelin receptor antagonist Ac-D-10,11-dihydro-5*H*-dibenzo[*a*,*d*] cycloheptene-glycine-3,3-D-diphenyl-Ala-Leu-Asp-Ile-Ile-Trp (PD142893) and it was only partially reduced by the endothelin ET_A receptor-selective endothelin antagonist PD151242.

Keywords: Endothelin ET_A receptor; Endothelin ET_B receptor; Fibroblast; (Human); HeLa cell

1. Introduction

Endothelin-1, which is expressed abundantly in endothelial and in vascular smooth muscle cells, provokes the contraction of vascular smooth muscle, as well as cardiac inotropism and proliferation (Haynes and Webb, 1998). Both the expression of endothelin-1 and its modulatory effects in vascular smooth muscle are mediated by two subtypes of receptors (ET_A and ET_B) (Alexander and Peters, 1999). In patients with coronary atherosclerosis, there is increased endothelin-1-like immunoreactivity in plasma (Timm et al., 1995) and peripheral vasoconstriction and decreased endothelin ET_B receptor expression have been observed in patients with congestive heart failure (Kobayashi et al., 1998). However, endothelin ET_B receptor-mediated endothelin-1 expression and vasodilator responses are often reported as being variable (Davenport et al., 1998). In earlier studies, we revealed the specific endothelin ET_B receptor-selective down-regulation of endothelin receptors to be a dominant mechanism in normal cells (Drimal et al., 1999). The present study with cultured normal and neo-plastic human cells describes distinct, intensified endothelin ET_B receptor-selective down-regulation of surface-membrane endothelin-1 receptors in neoplastic cells as a plausible mechanism leading to cell pathology.

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2. Methods

(3-[^{125}I]Tyr^{13})Endothelin-1, specific activity 2200 Ci/mmol; [^{125}I]Tyr^{13}-Suc-[Glu^{9}, Ala^{11,15}]-endothelin-1-(8–21) ([^{125}I]IRL-1620), specific activity 2000 Ci/mmol; (3-[^{125}I]-D-Tyr^{3})-(*N*-[(*N*-hexahydro-1*H*-azepin-1-yl)carbonyl]-L-Leu^{1}]-1-Me-D-Trp^{2}]) ([^{125}I]-PD151242), specific activity 2200 Ci/ mmol; (all from NEN Life Sci. Products, Boston, MA, USA); [^{3}H]thymidine, specific activity 29 Ci/ mmol (ICN); *N*-Acetyl-[Ala^{11,15}](6-21)endothelin-1 (BQ3020); (*N*-[*N*-[*N*[(2,6-dimetyl-1-piperidinyl)-carbonyl]-4 -methyl -L- Leu]- 1-(methoxy-carbonyl)-D-Trp]-D-norleucine-monosodium) (BQ788); (PD151242) (all from RBI); endothelin-1 (Sigma); IRL1620 (ICN). Human fibroblasts and HeLa cells used in this study came from the Institute of Virology (SAS). The cell lines were routinely grown as monolayer cultures in minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum, non-essential aminoacids, and 100 U/ml of each penicillin and streptomycin, at 37°C, in atmosphere of 5% CO_2/ 95% air. The experiments were performed with cells synchronized by incubation with low serum (1% fetal bovine serum, 72 h). Equilibrium binding and competition studies with human cells were carried out at 37°C in 250-μl volumes containing HEPES-buffered physiological salt solution with [^{125}I]endothelin-1 and in further experiments with [^{125}I]IRL-1620 or [^{125}I]PD151242. The human cells in culture, whole-cell ligand binding and assays of [^{3}H]thymidine incorporation have been described in detail previously (Drimal and Koprda, 1996; Drimal et al., 1999).

3. Results

The pre-replicative period of both normal human fibroblasts and tumorigenic HeLa cells was slightly reduced by increasing the cell density and was unaffected by changing the serum concentration. Both types of cells used in the present study showed similar responses in terms of binding affinity and the maximal number of endothelin-1 receptors. Saturation experiments with two subtype-selective endothelin receptor ligands, i.e. [^{125}I]PD151242 and [^{125}I]IRL-1620, showed proportional distribution of both subtypes of endothelin receptors in human fibroblasts and overexpression of endothelin ET_A receptors (1.45: 1, $P < 0.05$) on HeLa cells. In order to estimate the effect of endothelin receptor agonists on human cell proliferation, we measured the incorporation of [^{3}H]thymidine. Under the present conditions, the incorporation of labeled thymidine increased linearly up to 24 h of incubation and radioactivity in both cell strains in the cell layer reached a plateau 8 h after the onset of incubation (data not shown).Competition experiments with human fibroblasts revealed that [^{125}I]endothelin-1 (0.85 nmol/l) binding was inhibited with the following order of potency: endothelin-1 > PD151242 ≫ BQ788 > sarafotoxin, and [^{125}I]IRL-1620 binding was inhibited with the following order of potency: BQ788 > sarafotoxin ≫ PD151242. Equilibrium binding studies were accomplished with three radioligands in control cells and in cells pre-incubated with different endothelin receptor agonists. The control maximal values of specific binding and binding affinity for [^{125}I]endothelin-1, the natural helical peptide, were $B_{max} = 456 \pm 22$ fmol/mg of protein and affinity was in the nanomolar range ($K_d = 0.41 \pm 0.01$ nmol/l) in human fibroblasts. Comparable with these values were also the characteristics of [^{125}I]endothelin-1 binding in HeLa cells, $B_{max} = 405 \pm 15$ and $K_d = 0.20 \pm 0.2$ nmol/l. The truncated, linear-type peptide [^{125}I]IRL-1620 bound to human fibroblasts with $B_{max} = 424 \pm 12$ fmol/mg of protein and with high affinity ($K_d = 0.15 \pm 0.1$ nmol/l), and to HeLa cells with lower density ($B_{max} = 289 \pm 115$ fmol/mg of protein, $P < 0.05$),

Table 1
The intensity of down-regulation of endothelin receptors on cultured normal and tumorigenic human cells

Cell line	Ligand	Preference (selectivity)	Prolonged cell stimulation with					
			Endothelin-1			BQ3020		
			D	A	P	D	A	P
Human	[^{125}I]ET-1	($ET_A \gg ET_B$)	84 ± 6	156 ± 11	145 ± 5	67 ± 15	132 ± 27	125 ± 7
Fibroblasts[1]	[^{125}I]IRL1620	($ET_B \gg ET_A$)	82 ± 8	118 ± 23	122 ± 6	66 ± 22	148 ± 20	115 ± 4
HeLa	[^{125}I]ET-1	($ET_A \gg ET_B$)	90 ± 5	167 ± 22	138[a] ± 9	63 ± 6	220 ± 22	114 ± 4
Cells[2]	[^{125}I]IRL1620	($ET_B \gg ET_A$)	43[a] ± 9	149 ± 35	132[a] ± 4	27[a] ± 14	114 ± 7	109 ± 5

With the nonselective human endothelin-1 and with the endothelin ET_B receptor-selective, truncated endothelin-1-(6–21) analogue, linear peptide BQ3020. Characteristics of binding of radioactive ligands [^{125}I]endothelin-1 with preferential affinity for endothelin ET_A receptors ($ET_A \gg ET_B$) and truncated (endothelin-1-(8–21) ligand [^{125}I]IRL-1620, with preferential affinity for endothelin ET_B receptors ($ET_B \gg ET_A$).

Measured parameters: (D) Surface endothelin receptor density; (A) Affinity, expressed here as K_d; and (P) Proliferative response ([^{3}H]thymidine incorporation) are expressed as percentages of control. Values are Means ± S.E.M., ($n = 24$).

[1] Non-tumorigenic human fibroblasts.

[a] Significant change compared with control, $P < 0.05$.

[2] HeLa cells (established from human colorectal carcinoma) after prolonged stimulation with two different endothelin receptor agonists (both in 0.1 nmol/l).

and with very low affinity ($K_d = 3.3 \pm 1.5$ nmol/l, $P < 0.05$). The association kinetics of [^{125}I]IRL-1620 were rapid, with the association constant k_1 being similar to that of [^{125}I]endothelin-1. Binding was reversible and analysis of dissociation showed that [^{125}I]IRL-1620 dissociated from a single site with $k_{-1} = 0.0044 \pm 0.0005$ min^{-1} ($n = 4$); the K_d calculated from these experiments was $0.17 \pm .0.04$. The changes in density, affinity and proliferation induced after pre-incubation of the two endothelin receptor agonists endothelin-1 and BQ3020 are summarized in Table 1. Pre-incubation with an equimolar concentration of the endothelin ET_B receptor-selective BQ3020-(6–21) analogue endothelin-1, produced a more profound down-regulation of endothelin-1 receptors on cultured cells than did endothelin-1. Analysis of the specific binding of the endothelin ET_B receptor-selective, truncated endothelin-1 analogue [^{125}I]IRL-1620 in HeLa cells indicated significantly decreased endothelin ET_B receptor density and significant reduction in affinity. The pre-incubation of HeLa cells with endothelin-1 or with BQ3020 in other experiments produced a further down-regulation of endothelin ET_B receptors ($B_{maxBQ3020} \ll B_{maxEndothelin-1}$) and facilitated the proliferative response of HeLa cells. Binding studies were performed also with human fibroblasts pre-incubated (1 h) with different specific endothelin ligands (in concentration of 0.1 and 1.0 nmol/l) and enzyme inhibitors (1.0 and 5.0 μmol/l, 2 h). In these experiments, the specific binding of [^{125}I]endothelin-1 declined in the order: BQ788 ≥ chloroquine ≫ PD-151242 ≫ Control ≥ phosphoramidon, and the corresponding affinity diminished as follows: control > phosphoramidon ≥ PD151242 > chloroquine > BQ788. The pre-incubation of human fibroblasts with BQ788, chloroquine or Ac-D-10,11-dihydro-5*H*-dibenzo[*a*,*d*] cycloheptene-glycine-3,3-D-diphenyl-Ala-Leu-Asp-Ile-Ile-Trp (PD142893) increased the relative density of high-affinity [^{125}I]endothelin-1 binding sites, and pre-incubation of human fibroblasts and HeLa cells with BQ3020 or endothelin-1 significantly reduced the relative density of low-affinity [^{125}I]endothelin-1 binding sites. The reduction in relative density of [^{125}I]IRL1620 binding sites in human fibroblasts pre-incubated with BQ3020 and with endothelin-1 in other experiments was comparable with that in the [^{125}I]endothelin-1 group; however, the decrease in relative affinity was much more pronounced in the human fibroblasts pre-incubated with endothelin-1.

4. Discussion

By using cell synchronization, [^{3}H]thymidine incorporation, radioligand binding techniques, and pharmacological characterization, we have been able to identify the subtypes of endothelin receptors and their down-regulation in cultured normal human fibroblasts and in neoplastic Hela cells. Our data showed that human fibroblasts and also HeLa cells responded to nanomolar concentrations of exogenous human endothelin-1 or to its truncated analogue BQ3020 with down-regulation of endothelin ET_B receptors and with mild proliferation. The most striking difference between human fibroblasts and HeLa cells in the present study was the more pronounced increase in total number of endothelin ET_A receptors and the decrease in total number of specific membrane-bound endothelin ET_B receptors on HeLa cells. This effect was even more pronounced after their exposure to endothelin ET_B receptor-selective BQ3020, producing nearly complete endothelin ET_B receptor-selective down-regulation. One possible explanation of these results is that endothelin ET_B receptors are already down-regulated in human neoplastic cells as a result of stimulation with angiogenic and growth factors produced by enhanced pericellular proteolysis in neoplastic cells (Werb, 1997). A second possibility is that, upon transformation of HeLa cells by oncogenes, aberrant cell signaling leads to altered endothelin ET_A receptor gene expression, resulting in ET_A receptor predominance and in potentially striking differences in morphology of tumorigenic cells (Burgering and Boss, 1995). A third possibility is that the endothelin ET_A receptors on human fibroblasts and more so on the HeLa cells, are post-translationally modified (subtype-ET_A preference, reduced down-regulation at increased affinity of [^{125}I]endothelin-1 binding sites in the present study) and, as a consequence, endothelin receptors on human fibroblasts and HeLa cells are less likely to bind restrained endothelin ET_B receptor agonists. While the first possibility seems acceptable, the relevance of the later two possibilities is questionable. It is very plausible that, upon the transformation and progression of cells to the invasive phenotype, pathological signaling from the dominant endothelin ET_A receptor subtype may overcome inhibitory signaling from the down-regulated endothelin ET_B receptor subtype. As a consequence, tumorigenic cells may become less responsive to the regulatory signals derived from their immediate microenvironment. It is noteworthy that in several types of tumor cells (such as colon adenocarcinomas and breast tumors) the source of extracellular matrix proteases, a possible modulatory factor producing vasoactive peptides, is not tumor cell, but cells that are in contact with tumor cells, i.e. stromal fibroblasts (Seuwett and Dan, 1996). Is the progression of cells to the invasive endothelin ET_A receptor-mediated phenotype a limiting factor that in itself may overcome the regulation of silent transcription endothelin genes in cells? So far, there is no direct information providing answers to these questions. Certain implications of potential value for clinical practice can be inferred from the present findings, since the data show that endothelin ET_A receptor-selective endothelin receptor antagonists may offer selective protection against the proliferation of tumor cells.

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