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European Journal of Pharmacology 344 (1998) 307–312

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Change of endothelin receptor subtype in the MEG-01 human megakaryoblastic cell line

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Received 4 November 1997; revised 8 December 1997; accepted 12 December 1997

Abstract

The aim of this investigation was to determine whether the endothelin receptor subtype of a megakaryoblastic cell line (MEG-01) changes during culture passages as cells undergo maturation and differentiation. On early-passage cells, binding of [^{125}I]endothelin-1 was completely inhibited by 1 μM BQ 123 (*cyclo*-[D-tryptophanyl-D-aspartyl–prolyl-D-valyl–leucyl]), but not by sarafotoxin 6C. Also the endothelin-1-enhancing effect on $[Ca^{2+}]_i$ was prevented by BQ 123, whereas sarafotoxin 6C had no effect on $[Ca^{2+}]_i$. In late-passage cells, endothelin ET_B analogs, unlike endothelin ET_A analogs, competed with binding of [^{125}I]endothelin-1. Endothelin ET_B receptor agonists increased $[Ca^{2+}]_i$ while the endothelin-1-induced response was inhibited by BQ 788 ([*N*-[(2*R*,6*S*)-2,6-dimethyl-piperidinocarbonyl]-4-methyl-D-leucyl]-[N^{ω}-(methoxycarbonyl)-D-tryptophanyl]-D-norleucine), but not by BQ 123, although both endothelin ET_A and ET_B receptor mRNAs were expressed, as shown by reverse transcriptase–polymerase chain reaction. These results demonstrate that in MEG-01 cells switch from expression of endothelin ET_A to expression of ET_B receptors during culture. The data also suggest that late-passage MEG-01 cells look like platelets, in terms of endothelin receptor subtype. © 1998 Elsevier Science B.V.

Keywords: Endothelin receptor subtype; MEG-01 cell line; Reverse transcriptase–polymerase chain reaction; Platelet

1. Introduction

Endothelins are a family of three 21-amino acid peptides produced by a wide variety of cell types which exert pleiotropic effects on many cell targets through at least two receptor subtypes, endothelin ET_A and ET_B receptors. These receptors are characterized by different sensitivity to endothelin isoforms and different affinity for natural and/or synthetic agonists and/or antagonists (for reviews, see Yanagisawa and Masaki, 1989; Simonson and Dunn, 1991; Goto et al., 1996).

In a previous paper (Diochot et al., 1992), we have shown that the human MEG-01 cell line, which may be considered as a good model of immature megakaryocytes, the progenitors of blood platelets (for review, see Avraham, 1993), is sensitive to endothelin isoforms. Indeed endothelin-1 induced in these cells both increases in intracellular ionized calcium concentration ($[Ca^{2+}]_i$) and inositol trisphosphate levels. The increase in $[Ca^{2+}]_i$ evoked by endothelin-1 was mimicked by other endothelin isoforms. Their rank order of potency was endothelin-1 > endothelin-3, which led us to class the endothelin receptor present on MEG-01 cells as an endothelin ET_A receptor subtype. However, in subsequent experiments performed on later passaged cells, which correspond to a more differentiated stage, we confirmed the $[Ca^{2+}]_i$-enhancing effect of endothelin isoforms, but could no longer find the rank order of potency to be endothelin-1 > endothelin-3. To complete the investigation of the expression of endothelin receptors present on these cells during their differentiation, we performed: 1 – studies investigating the competition of binding of radiolabelled endothelin-1 by the selective endothelin receptor molecular probes, in particular the endothelin ET_A selective receptor antagonist BQ 123 (*cyclo*-[D-tryptophanyl-D-aspartyl–prolyl-D-valyl–leucyl]) (Ihara et al., 1992), the selective endothelin ET_B receptor agonists IRL 1620 ([9-*N*-succinyl–glutamic acid, 11-alanine, 15-alanine]endothelin-1_{8-21}) (Karaki et al., 1993), sarafotoxin 6C (Williams et al., 1991) and antagonist BQ 788 ([*N*-[(2*R*,6*S*)-2,6-dimethyl–piperidinocarbonyl]-4-

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methyl-D-leucyl]-[N^{ω}-(methoxycarbonyl)-D-tryptophanyl]-D-norleucine) (Ishikawa et al., 1994), 2 – functional studies on the effect of endothelin analogs on $[Ca^{2+}]_i$ and 3 – studies investigating the expression of messenger RNA coding for the two subtypes of endothelin receptors, using reverse transcriptase–polymerase chain reaction (RT-PCR). These investigations were performed on early- and/or late-passage MEG-01 cells.

2. Materials and methods

2.1. Materials

The MEG-01 cell line was provided by Pr H. Saito, Nagoya, Japan. RPMI 1640 was from Gibco and fetal calf serum from Flow Laboratory. Endothelin-1 and -3, IRL 1620 and sarafotoxin 6C were from Alexis (Switzerland). BQ 123 and BQ 788 were kindly given by Mrs. S. Jouquey (Roussel-Uclaf, France).

Indo-1 acetomethyl ester was purchased from Calbiochem (France) and [^{125}I]endothelin-1 (2200 Ci/mmol) was from DuPont N.E.N. (France). The oligonucleotides used were synthesized by Eurogentec (Belgium). Moloney Murine Leukemia Virus (MMLV) reverse transcriptase was from Gibco (France), human placental ribonuclease inhibitor was from Amersham (France), hexamers were from Boehringer-Inghelheim (France) and Taq DNA polymerase was from Promega (France). All other reagents were from Sigma (USA).

The composition of the phosphate-buffered saline (PBS) used for Ca^{2+} measurements was (mM) NaCl, 138; KCl, 2.7; KH_2PO_4, 1.47; Na_2HPO_4, 8; $MgCl_2$, 0.5; $CaCl_2$, 0.9; glucose, 5.55. It was adjusted to pH 7.4. For binding experiments the same medium plus 2 mg/ml bovine serum albumin and 1 mg/ml bacitracin was used.

2.2. Cell culture

Cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 1 mM glutamine, 1% sodium pyruvate and 0.4% non-essential aminoacids. They were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and routinely passaged by 50% dilution of cell suspension with fresh medium every 3 days. Early-passage cells were those of the passages 10–40 after receipt of the cells and late-passage cells were those of passages 60–90.

2.3. RT-PCR procedure

Total RNA were extracted from late-passage MEG-01 cells by using a guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). After denaturation (70°C, 5 min) of 1.5 μg of total RNA with 1 μg hexamers, first cDNA was synthesized by using 200 U MMLV reverse transcriptase, 17.5 U human placental ribonuclease inhibitor, 10 mM dithiothreitol and 0.6 mM of each dNTP in 25 μl buffer. The mixture was incubated for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min. A blank was prepared without reverse transcriptase.

The amplification of cDNA was carried out in a final volume of 50 μl. The specific oligonucleotides for the human endothelin receptor cDNA sequence (Elshourbagy et al., 1993) used for RT-PCR were (sequences written from 5′ to 3′)

Endothelin ET_A receptor subtype: forward CCTTTTGATCACAATGACTTT (bp 439–459), backward TTTGATGTGGCATTGAGCATACAG (bp 737–714).

Endothelin ET_B receptor subtype: forward ACTGGCCATTTGGAGCTGAGAT (bp 497–518), backward CTGCATGCCACTTTTCTTTCTCAA (bp 924–901).

2 μl of each reverse transcriptase reaction product was added to *Taq* buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 μM of each oligonucleotide for ET_A or 0.25 μM for ET_B and 4 U *Taq* DNA polymerase. Samples were heated in a thermal cycler (Biometra) to 94°C for 2 min then 30 cycles were conducted in three temperature steps: 30 s at 57°C, 30 s at 72°C and 30 s at 94°C (endothelin ET_A receptor), or 30 s at 65°C (for both annealing and synthesis) and 30 s at 94°C (endothelin ET_B receptor). After the last annealing step, the samples were incubated at 72°C for 3 min to achieve primer extension. The expected sizes of the PCR products were 299 and 428 bp for endothelin ET_A and ET_B receptors, and comprised 1 (> 4 kb) and 2 (2 kb) introns, respectively.

Aliquots of each PCR reaction mixture were submitted to electrophoresis in 1.5% agarose gel containing ethidium bromide for visualization.

2.4. Radioligand binding and competition studies

The binding of peptides to intact MEG-01 cells was measured by competition with [^{125}I]endothelin-1. Experiments were performed in a final volume of 0.2 ml PBS containing glucose, bacitracin and bovine serum albumin. Cells $3 \cdot 10^4$ were incubated with 4 pM [^{125}I]endothelin-1. Unlabelled 1 μM endothelin-1 was used to determine nonspecific binding. With early-passage cells, competition studies were performed with 1 μM BQ 123 or sarafotoxin 6C. For competitive binding assays with late-passage cells, various concentrations of endothelin-1 and -3, BQ 788 and BQ 123 (0.1 to 1000 nM) were used. Incubations were performed at 4°C for 1 h 30 min. The reaction was terminated by filtration under vacuum through Whatman Glass GF/C filters which where rinsed 3 times with 4 ml ice-cold PBS buffer and the radioactivity bound to the filters was counted in a gamma-spectrometer.

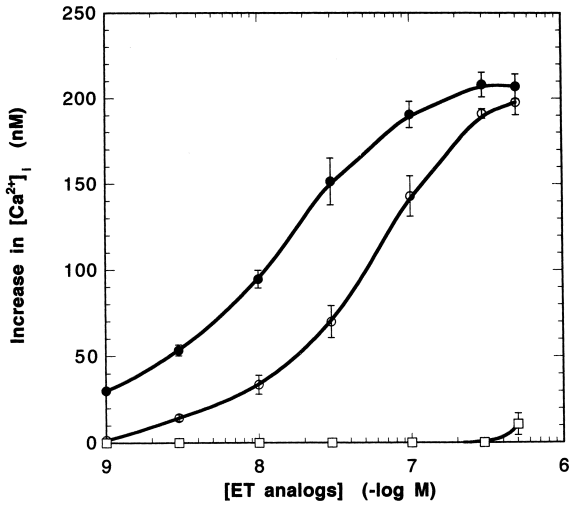

Fig. 1. Concentration–response curves for the increase in intracellular ionized calcium ($[\Delta Ca^{2+}]_i$) elicited by endothelin-1 (●), sarafotoxin 6C (□), and endothelin-1, added 5 min after 1 μM BQ 123 (○), in-early passage MEG-01 cells. Each curve represents the mean of 4 individual experiments. Bars are S.E.M.

2.5. Measurement of $[Ca^{2+}]_i$

Early- and late-passage MEG-01 cells were washed twice with PBS-glucose, resuspended at 10^6 cells/ml in the same medium and incubated 20 min at 37°C in the dark with 5 μM indo-1 acetomethylester. Loading was terminated by dilution with cold PBS-glucose, followed by centrifugation. The cell pellet was resuspended in PBS-glucose and aliquots ($1 \cdot 10^6$ cells) were transferred to a 1-ml quartz cuvette equipped for temperature control and magnetic stirring. Fluorescence signals were obtained as indicated in our previous paper (Diochot et al., 1992), using a Kontron SFM 25 spectrofluorimeter. The concentration of ($[Ca^{2+}]_i$) was calculated according to Grynkiewicz et al. (1985).

All agents were added to the quartz cuvette in a volume of 10 μl. Endothelin receptor antagonists were given to cells 5 min before the endothelin receptor agonists.

2.6. Data analysis

All values given are mean values ± S.E.M. EC_{50} values for radioligand binding experiments were analyzed by using the computerized program LIGAND.

3. Results

3.1. Experiments on early passage MEG-01 cells

The specific binding of [^{125}I]endothelin-1 to cells was completely inhibited by 1 μM BQ 123 (3 ± 4%, *n*: 4), while 1 μM sarafotoxin 6C had no effect (94 ± 10%, *n*: 4).

BQ 123 (1 μM) caused a rightwards shift of the concentration–response $[Ca^{2+}]_i$ curve for endothelin-1, without causing any significant modification of the maximal response whereas sarafotoxin 6C had no effect (Fig. 1).

3.2. Experiments on late passage MEG-01 cells

3.2.1. Analysis of the RT-PCR products

Following the RT procedure, the PCR-amplified products of cDNA were of the sizes predicted for endothelin receptor subtype ET_A (299 bp) and endothelin receptor subtype ET_B (428 bp). In the absence of reverse transcriptase no products were formed (Fig. 2).

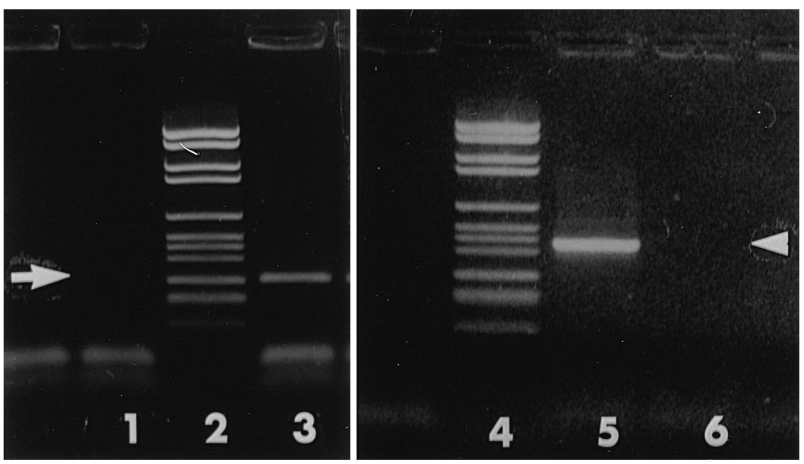

Fig. 2. Analysis of PCR-amplified cDNA from late-passage MEG-01 cells. RT-PCR was performed as described in Section 2 and the products were analyzed on 1.5% agarose gels. Lanes 2 and 4: molecular weight markers; lane 3: endothelin ET_A receptor mRNA; lanes 1 and 6: RT-PCR without reverse transcriptase; lane 5: endothelin ET_B receptor mRNA. The sizes of the amplified products (299 and 428 bp for endothelin ET_A and ET_B receptor subtypes, respectively) are indicated with an arrow (endothelin ET_A receptor) and an arrow head (endothelin ET_B receptor).

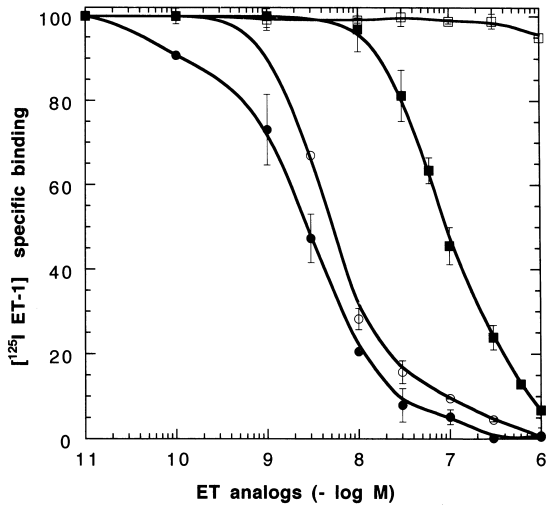

Fig. 3. Competition curves of selected endothelin receptor agonists and antagonists for inhibition of [^{125}I]endothelin-1 binding to MEG-01 cells: endothelin-1 (○), endothelin-3 (□), BQ 788 (●) and BQ 123 (■). Results are from five or six independent experiments performed in duplicate. Bars are S.E.M.

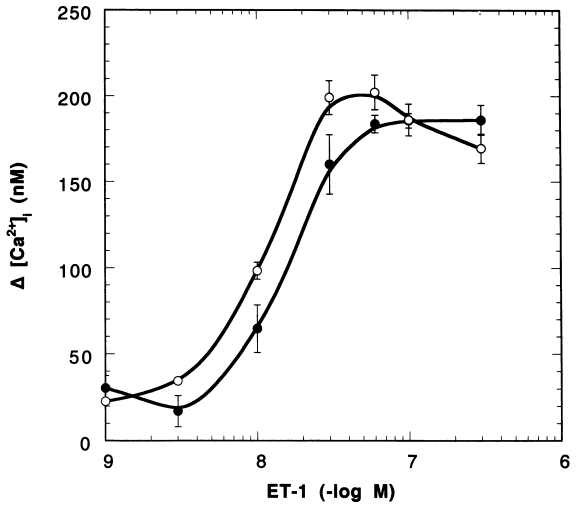

Fig. 5. Concentration–response curve for the increase in intracellular ionized calcium ($[\Delta Ca^{2+}]_i$) elicited by endothelin-1 (○) and for endothelin-1, added 5 min after 10 μM BQ 123 (●), in late-passage MEG-01 cells. Each curve represents the mean of 4 individual experiments. Bars are S.E.M.

3.2.2. Competitive binding experiments

The specific binding of [^{125}I]endothelin-1 was inhibited in a concentration-dependent fashion by endothelin-1 and -3 and by BQ 788, ($IC_{50} = 3.75 \pm 0.65$, 7.62 ± 1.04 and 120 ± 28 nM, for endothelin-1, -3 and BQ 788, respectively), with complete inhibition at 1 μM. In contrast, the binding of [^{125}I]endothelin-1 was not attenuated ($EC_{50} \gg 10$ μM) by the endothelin ET_A receptor-selective antagonist BQ 123 (Fig. 3).

3.2.3. Functional studies

The endothelin ET_B receptor-selective agonists sarafotoxin 6C and IRL 1620 increased $[Ca^{2+}]_i$ in Indo-1-loaded MEG-01 cells, in a concentration-dependent manner (Fig. 4). BQ 123 (10 μM) did not produce any significant modification in the concentration–effect curve for the increase in $[Ca^{2+}]_i$ by endothelin-1 (Fig. 5) whereas BQ 788 decreased that effect in a concentration-dependent manner, with complete inhibition at 1 μM (Fig. 6).

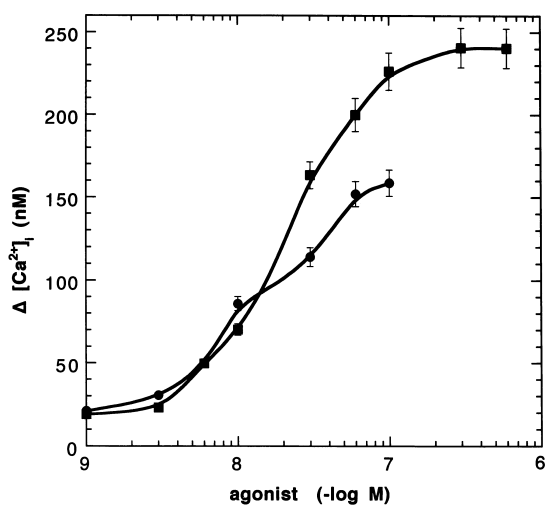

Fig. 4. Concentration–response curves for the increase in intracellular ionized calcium ($[\Delta Ca^{2+}]_i$) elicited by sarafotoxin 6C (■) and IRL 1620 (●) in late-passage MEG-01 cells. Each curve represents the mean of 4 individual experiments. Bars are S.E.M.

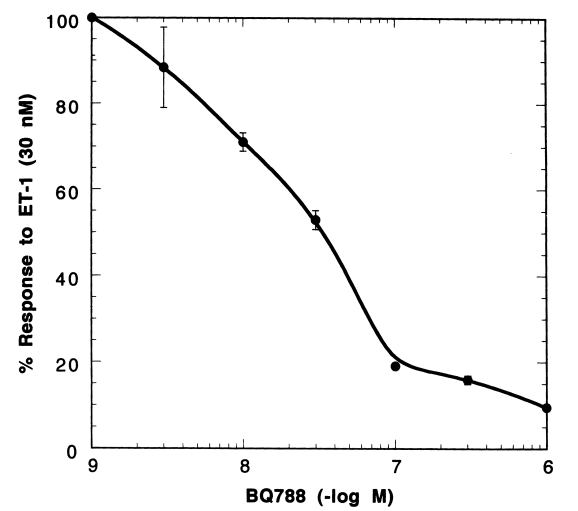

Fig. 6. Concentration–response curve for BQ 788 on the 30 nM endothelin-1-evoked increase in intracellular ionized calcium ($[\Delta Ca^{2+}]_i$) in late-passage MEG-01 cells. Each curve represents the mean of 4 individual experiments. Bars are S.E.M.

4. Discussion

Our previous findings (Diochot et al., 1992) showed that in MEG-01 cells endothelin isoforms increased $[Ca^{2+}]_i$ according to the rank order of potency, endothelin-1 > endothelin-3, suggesting the involvement of endothelin ET_A receptors in these responses. Such an involvement was confirmed by the observation reported here that BQ 123, a selective endothelin ET_A receptor antagonist, was able to completely inhibit binding of radiolabelled endothelin-1 to cells and caused a rightwards shift of the concentration–response Ca^{2+} curve for endothelin-1 in early-passage MEG-01 cells. The lack of endothelin ET_B receptors on these cells was shown by the lack of effect of sarafotoxin 6C on [^{125}I]endothelin-1 binding and $[Ca^{2+}]_i$.

The situation was quite different in cells at later passages. Indeed, RT-PCR experiments showed that both endothelin ET_A and ET_B receptor mRNA were expressed in these cells. Nonetheless, in pharmacological experiments, BQ 123 was neither able to compete with radiolabelled endothelin-1 binding to cells, nor to modify the concentration–response curve for the endothelin-1-induced increase in $[Ca^{2+}]_i$. These data then suggest that the endothelin ET_A receptor mRNA of late-passage cells was not translated to endothelin ET_A receptor protein able to recognize BQ 123.

In contrast, endothelin ET_B receptor mRNA was translated to endothelin ET_B receptor protein. Indeed, the rank order of potency of endothelin isoforms for competing with radiolabelled endothelin-1 binding to these cells was endothelin-1 = endothelin-3. Moreover, BQ 788, a selective endothelin ET_B receptor antagonist, was able to compete with [^{125}I]endothelin-1 binding to late-passage MEG-01 cells. However, the binding parameters reported here, and in particular for BQ 788, are higher those obtained for cell lines transfected with human or bovine endothelin receptors (Bax and Saxena, 1994). These discrepancies could be related to the fact that MEG-01 cells express and release endothelin-1 (Mathieu et al., 1997), leading to desensitization of receptors and to an altered functional response to various endothelin peptides. Such a hypothesis is probably true for the ET_A- or ET_B-induced activation of endothelin receptors in early- or late-passage MEG 01 cells.

Like the ET_A endothelin receptors on early-passage cells, the endothelin ET_B binding sites found on late-passage MEG 01 cells are functional endothelin ET_B receptors since on the one hand both the endothelin ET_B receptor-selective agonists sarafotoxin 6C and IRL 1620 were able to increase $[Ca^{2+}]_i$ in these cells in a concentration-dependent fashion and on the other hand the $[Ca^{2+}]_i$ response evoked by endothelin-1 was inhibited in a concentration-dependent manner by BQ 788, with complete inhibition at 1 μM inhibitor.

Taken together, our previous and present findings show that in early passages, the MEG-01 cell line only expresses functional endothelin ET_A receptors and that in later passages it expresses both endothelin ET_A and ET_B receptor mRNAs, although only the endothelin ET_B receptor mRNA seems to be translated, processed, and targeted to cell membrane as a functional receptor. A similar finding has already been described (Eguchi et al., 1994). In their study performed with cultured rat vascular smooth muscle cells, these authors described a change in endothelin receptor subtype from endothelin ET_A to ET_B receptor, during transition from early to late passage, a change which was associated with a marked modification of the endothelin isoform-evoked mitogenic effects.

The MEG-01 cell line has phenotypical properties of megakaryoblasts (Ogura et al., 1985), the immediate precursors of megakaryocytes (Avraham, 1993). It has been shown that treatment of the clonal MEG-01 cell line with 12-*O*-tetradecanoylphorbol-13 acetate or phorbol-12,13-butyrate causes cells to have differentiation markers of mature megakaryocytes, with increased ploidy (Ogura et al., 1988). In addition, even in the absence of treatment, MEG-01 cells are able to produce platelet-like particles (Takeuchi et al., 1991). Our personal experience (unpublished observations) is that this production occurs essentially in late-passage MEG-01 cells, which appear more mature than early-passage cells. The change in the endothelin receptor subtype in MEG-01 cells from endothelin ET_A to ET_B receptor subtype, found in early- and late-passage cells, respectively, also appears to correspond to a more mature phenotype in the megakaryoblast–megakaryocyte–platelet lineage. Indeed, when considering the endothelin receptor subtype of platelets, it has been shown that endothelin-3 (Gagnet et al., 1996) as well as endothelin-1 and sarafotoxin 6C (Dockrell et al., 1996) increase the cGMP content in human blood platelets. From these results, it is possible to conclude that platelets bear endothelin ET_B receptors, whose activation leads to increased cGMP and therefore contributes to inhibition of platelet aggregation.

Our findings show that there was a phenotypic modification in MEG-01 cells during changes from early to late passages, corresponding to a change from endothelin ET_A to ET_B receptor subtype. It appears that this change reflects a transition to more mature cells that are able to produce platelet-like particles, and which resemble human platelets in their endothelin receptor subtype.

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