Endothelin-induced Apoptosis of A375 Human Melanoma Cells*

Makoto Okazawa‡‡, Takuma Shiraki‡‡, Haruaki Ninomiya, Shigeo Kobayashi‡, and Tomoh Masaki†

From the Department of Pharmacology, Faculty of Medicine, and ‡Department of Neuroscience, Graduate School of Human and Environmental Studies, Kyoto University, Kyoto 606, Japan

Endothelin-1 (ET-1) inhibited serum-dependent growth of asynchronized A375 human melanoma cells, and the growth inhibitory effect was markedly enhanced when ET-1 was applied to the cells synchronized at G1/S boundary by double thymidine blocks. Flow cytometric analysis revealed that ET-1 did not inhibit the cell cycle progression after the release of the block but caused a significant increase of the hypodiploid cell population that is characteristic of apoptotic cell death. ET-1-induced apoptosis was confirmed by the appearance of chromatin condensation on nuclear staining and DNA fragmentation on gel electrophoresis. The increase in the hypodiploid cell peak was manifest within 16 h of exposure to 5 nM ET-1. Within the same time range, ET-1 caused actin reorganization and drastic morphological changes of the surviving cells from epithelioid to an elongated bipolar shape. These phenotypical changes were preceded by ET-1-induced increase and nuclear accumulation of the tumor suppressor protein p53. All of these effects of ET-1 were mediated by ETB receptors via a pertussis toxin-sensitive G protein. Flow cytometric analysis with fluorescent dye-labeled ET-1 revealed up-regulation of ETB receptors by the cells in G1/early S phases, and overexpression of the receptor protein by cDNA microinjection conferred the responsiveness (both apoptosis and morphological changes) to ET-1 irrespective of the position of the cell in the cell cycle. These results indicated the presence of ETB-mediated signaling pathways to apoptotic cell machinery and cytoskeletal organization. Furthermore, the densities of ETB receptors expressed by individual A375 melanoma cells appeared to be regulated by a cell cycle-dependent mechanism, and the receptor density can be a limiting factor to control the apoptotic and cytoskeletal responses of the cells to ET-1. Although the molecular mechanisms remain to be elucidated, these findings added a new dimension to the diverse biological activities of ETs and also indicated a novel mechanism to control the responsiveness of the cell to the peptides.

The endothelin (ET) family of peptides include three isoforms, ET-1, ET-2, and ET-3 (1) and exert their effects by binding to specific G protein-coupled receptor (GPCR) subtypes, ETA and ETB (2, 3). Subsequent to the identification of ET-1 as a potent vasoconstrictive peptide (4), numerous studies described their diverse biological effects on target cells (reviewed in Ref. 5), and from these studies has emerged the recognition of ETs as one of the growth factors on definite cell lineages. ET-1 has been reported to stimulate DNA synthesis and proliferation of a variety of cells such as smooth muscle cells, glomerular mesangial cells, osteoblasts, fibroblasts, and mature melanocytes (reviewed in Ref. 6). Because of the predominant expression of ETA by these cell types and also because of the mitogenic signaling by ETA in transfected cells (7), it is generally accepted that ETA transmits a positive signal to the cell growth. In contrast, despite the diverse distribution of ETB both in tissues and cell lines, there are only a few reports in the literature that provided circumstantial evidence for the mitogenic signaling transmitted by ETB (8, 9). Furthermore, Mallat et al. (10) revealed ETB-mediated growth suppression of hepatic Ito cells in their myofibroblastic phenotype, demonstrating the ability of ETB to transmit a signal to the cell growth that is completely opposite to that transmitted by ETA.

Gene targeting revealed the critical role of ETB in embryonic development of melanocytes (11). Subsequent studies on primary cultured melanocyte precursor cells revealed multiple roles of ETB in growth/differentiation of the cells that apparently differ between the differentiation stages of the cells (12). In keeping with these findings, various human melanoma cell lines have been shown to express this receptor subtype (13, 14), which indicates the possibility that each melanoma is derived from the fully differentiated cell, melanocyte, and retains the characteristics of undifferentiated melanocyte-precursor cells.

Recently, two GPCRs for peptide ligands, namely angiotensin II receptor type 2 (ATII2) and somatostatin receptor 3 (SSTR3), have been shown to transmit growth inhibitory effects associated with programmed cell death or apoptosis of the target cells (15–17). Because of the critical involvement of apoptosis in the cellular differentiation (18), given the multiple roles of ETB on growth/differentiation of melanocyte-precursor cells and the heterogeneity of melanoma cell lines, we postulated that ETB may cause a growth inhibitory effect on some of the melanoma cell lines. In the initial screening on the three human melanoma cell lines A375, MeWo and HM3KO, we found that ET-1 caused a growth inhibitory effect only on A375. Therefore, the current study was conducted to characterize the growth inhibitory effect of ET-1 on this cell line.

The abbreviations used are: ET, endothelin; ETB, endothelin B; BSA, bovine serum albumin; [Ca2+]i, intracellular calcium concentration; FCS, fetal calf serum; GFP, green fluorescent protein; G protein, guanylic nucleotide-binding regulatory protein; GPCR, G protein-coupled receptor; hETB, human endothelin B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diaryl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; DMEM, Dulbecco's modified Eagle's medium; PTX, pertussis toxin; SSSTR3, somatostatin receptor type 3.

* This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan. Tel.: 81-75-753-4477; Fax: 81-75-753-4402; E-mail: masaki@mfour.med.kyoto-u.ac.jp.

‡ The first two authors made equal contributions to this work.

§ The abbreviations used are: ET, endothelin; ETB, endothelin B; BSA, bovine serum albumin; [Ca2+]i, intracellular calcium concentration; FCS, fetal calf serum; GFP, green fluorescent protein; G protein, guanylic nucleotide-binding regulatory protein; GPCR, G protein-coupled receptor; hETB, human endothelin B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diaryl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; DMEM, Dulbecco's modified Eagle's medium; PTX, pertussis toxin; SSSTR3, somatostatin receptor type 3.
Experimental procedures

Materials—Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were from Life Technologies Inc. (Tokyo, Japan); synthetic human ET-1 and ET-3, BQ123, and BQ788 were from the Peptide Institute (Osaka, Japan); [125I]ET-1 (74 TBq/mmol), [γ-32P]ATP (220 TBq/mmol), FluoroLink™ Cy2 Reactive Dye-Pack, and Cy2-avidin were from Amersham Pharmacia Biotech (Buckinghamshire, UK); EASYTAG™ Express Protein labeling mix [aS] (43.5 TBq/mmol) was from NEN Life Science Products (Tokyo, Japan); pertussis toxin (PTX) was from Funakoshi Co. (Tokyo, Japan); Texas Red-phalloidin was from Molecular Probes (Eugene, OR); mouse monoclonal anti-α-tubulin antibody was from MONOSAN (Uden, Netherlands); mouse monoclonal anti-p53 antibody (DO-1) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were of reagent grade and were obtained commercially.

Cell Culture—A375 human melanoma cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were routinely maintained in DMEM, 10% FCS at 37 °C in a humidified atmosphere containing 5% CO2. They were passaged twice a week at the ratio of 1:5 to keep an exponentially growing state. The substratum was cell culture grade plasticware (Costar, Cambridge, MA) except for the use of poly-L-lysine-coated glass-bottom culture dishes (MatTek Corp., Ashland, MA) for immunocytochemical experiments. Cell cycle synchronization was achieved by two sequential thymidine blocks according to the procedures described by Stein et al. (19). In brief, cells in the exponential growth phase were exposed to 2.5 mM thymidine in DMEM, 10% FCS for 16 h and then incubated in the fresh medium for 10 h. The cells were once again exposed to 2.5 mM thymidine for 16 h, and the block was released by replacing the medium to fresh DMEM, 10% FCS. Where indicated, the indicated concentrations. The cells were exposed to 400 ng/ml PTX during the last 2 h of the second thymidine block. As an alternative method of cell synchronization, a plant amino acid mimosine was used according to the procedures described by Krek and DeCaprio (20). In brief, cells were exposed to 0.1 mM mimosine in DMEM, 10% FCS for 18 h, and the block was released by replacing the medium with fresh DMEM, 1% FCS.

125I-ET-1 Binding Assay—Binding assays on attached cells were done according to the procedures described (3) with slight modifications. In brief, for saturation isotherms, cells at ~50% confluence in 48-well plates were washed with ice-cold phosphate-buffered saline (PBS) and then incubated at 4 °C for 60 min in 0.25 mM of PBS, 0.2% BSA containing increasing concentrations (5–300 pM) of 125I-ET-1. After washing the cells with PBS, the radioactivity associated with the cells was recovered at 0.1 N NaOH and counted in a γ-counter. Nonspecific binding was defined as the binding in the presence of 100 nM unlabeled ET-1 and was always less than 10% of the total binding capacity. For displacement experiments, the cells were incubated with 100 pM [125I]ET-1 and increasing concentrations (100 pM to 1 μM) of an unlabeled peptide.

Cell Proliferation Assay—Asynchronous or phase-synchronized cells at ~50% confluence in 96-well plates were incubated for 24 h in DMEM, 1% FCS containing increasing concentrations of ET-1. The number of viable cells in each well was estimated by measurement of the rate of mitochondrial metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) using a cell proliferation kit (Promega, Madison, WI) according to the manufacturer’s instructions.

Flow Cytometry—For cell cycle analysis, cells were washed with PBS, harvested by trypsinization, fixed in 70% ethanol, and then labeled with propidium iodide (PI) by incubation for 30 min at room temperature in PBS containing 50 μg/ml PI and 1 mg/ml ribonuclease A. The DNA content per nucleus was analyzed by a FACScan flow cytometer (Becton Dickinson Co., San Jose, CA). For p53 immunostaining, the harvested cells were fixed first in 1% paraformaldehyde and then in 70% ethanol. The cells were labeled with anti-p53 antibody followed by staining with fluorescein isothiocyanate-labeled goat anti-mouse IgG and then subjected to flow cytometry. For Cy2-ET-1 binding, cells were harvested by scraping and fixed in 100% methanol. After washing with PBS, 0.2% BSA, the cells were incubated for 30 min at room temperature in the same medium containing Cy2-ET-1 at the indicated concentrations. The cells were washed with PBS, 0.2% BSA, and 1% Tween and resuspension associated with each cell was analyzed. Cy2-ET-1 binding was prepared by using FluoroLink™ Cy2 Reactive Dye-Pack (Amersham Pharmacia Biotech) as described (21).

Immunocytochemistry—For Texas Red phalloidin/anti-α-tubulin double staining, cells in poly-l-lysine-coated glass-bottom dishes were washed with PBS, fixed in 3.7% formaldehyde/PBS at room temperature for 10 min, and permeabilized in acetone at ~20 °C for 10 min. The following procedures were done at room temperature. After blocking in PBS, 3% BSA, Texas Red-phalloidin and anti-α-tubulin antibody were simultaneously applied for 30 min in the same medium. The cells were further treated with biotinylated anti-mouse IgG followed by Cy2-avidin and then mounted in fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL). Fluorescent images of the cells were obtained with a MRC1024 laser-scanning confocal microscope (Bio-Rad, Osaka, Japan). For p53 immunostaining, fixed and permeabilized cells were stained with anti-p53 antibody in PBS for 30 min and then processed in exactly the same way. DNA staining with PI of the fixed cells were done as described above for the flow cytometry.

DNA Fragmentation Analysis—Isolation of DNA and analysis by agarose gel electrophoresis were done as described (22). In brief, cells were lysed by incubation at 50 °C for 12 h in the lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.2 mM proteinase K). DNA was extracted once with phenol/chloroform/isooamyl alcohol and twice with chloroform/isooamyl alcohol. After ethanol precipitation and resuspension, 10 μg of DNA samples were subjected to 1.2% agarose gel electrophoresis and visualized under UV light.

Metabolic Labeling of p53—During the last 7 h of the second thymidine block, the cells in 60-mm dishes were washed with PBS and, after a preincubation in methionine/cysteine (Met/Cys)-free DMEM for 20 min, were pulse-labeled for 1.5 h with [35S]Met/Cys (50 μCi/ml) in the same medium supplemented with 10% FCS. The cells were then chased
for 4.5 h in the same medium. All the medium used contained 2.5 mM thymidine to keep the cell cycle blocked. The cells were then incubated in DMEM, 1% FCS containing increasing concentrations of ET-1. At 24 h, the number of viable cells in each well was estimated by MTT assay. In separate experiments, the phase-synchronized cells were incubated in DMEM, 1% FCS containing 5 nM ET-1 with 1 μM BQ123 or BQ788 (bar graph). The cell growth was expressed as relative to that induced by 1% FCS (100%). Shown are the means ± S.E. of three determinations each done in triplicate. *, p < 0.05, and **, p < 0.01; significantly different from the values in the presence of 1% FCS. Arrow indicates the hypodiploid peak. c, time-dependent quantification of the cells with hypodiploid DNA. Phase-synchronized cells were incubated in DMEM, 1% FCS with or without 5 nM ET-1. At the times indicated, cells were harvested, and the DNA contents were analyzed. d, effects of ET receptor antagonist and PTX treatment. The cells were incubated for 24 h in h with or without ET receptor antagonist as indicated. The concentration of ET-1 was 5 nM and that of BQ123/BQ788 was 1 μM. In separate experiments, the cells were treated with PTX (400 ng/ml) during the last 2 h of the second thymidine block and then incubated for 24 h in DMEM, 1% FCS with 5 nM ET-1. c and d, the number of the hypodiploid cells were expressed as relative to the total number of cells analyzed. Shown are the means ± S.E. of three independent determinations. *, p < 0.01; significantly different from the control values.

**Fig. 2. Growth inhibition of A375 cells by ET-1 associated with apoptotic cell death.** a, growth inhibition assessed by MTT assay. Cells under asynchronous state (closed circles) or those phase-synchronized by double thymidine blocks (open circles) in 96-well plates were incubated either in serum-free DMEM or in DMEM, 1% FCS containing increasing concentrations of ET-1. At 24 h, the number of viable cells in each well was estimated by MTT assay. In separate experiments, the phase-synchronized cells were incubated in DMEM, 1% FCS containing 5 nM ET-1 with 1 μM BQ123 or BQ788 (bar graph). The cell growth was expressed as relative to that induced by 1% FCS (100%). Shown are the means ± S.E. of three determinations each done in triplicate. *, p < 0.05, and **, p < 0.01; significantly different from the values in the presence of 1% FCS. Arrow indicates the hypodiploid peak. c, time-dependent quantification of the cells with hypodiploid DNA. Phase-synchronized cells were incubated in DMEM, 1% FCS with or without 5 nM ET-1. At the times indicated, cells were harvested, and the DNA contents were analyzed. d, effects of ET receptor antagonist and PTX treatment. The cells were incubated for 24 h in h with or without ET receptor antagonist as indicated. The concentration of ET-1 was 5 nM and that of BQ123/BQ788 was 1 μM. In separate experiments, the cells were treated with PTX (400 ng/ml) during the last 2 h of the second thymidine block and then incubated for 24 h in DMEM, 1% FCS with 5 nM ET-1. c and d, the number of the hypodiploid cells were expressed as relative to the total number of cells analyzed. Shown are the means ± S.E. of three independent determinations. *, p < 0.01; significantly different from the control values.
RESULTS

Predominant Expression of ET_B by A375 Cells—Melanoma cell lines are classified according to the differentiation stages assessed by morphology, expression of cell-surface marker antigens, and pigmentation (24), and A375 cell line belongs to a relatively undifferentiated class because of the epithelioid shape, expression of HLA-DR antigen, lack of gp100 antigen, and lack of pigmentation. Saturation isotherms with 125I-ET-1 revealed that, under the routine culture conditions, the cells express ET-1 binding sites with $K_d$ values of 31 ± 6 pm and $B_{max}$ values of 3.2 ± 0.1 × 10^3 per cell (means ± S.E., n = 3) (Fig. 1a). The complete and partial displacements of the binding by BQ788 (an ET_B-selective antagonist; Ref. 25) and BQ123 (an ET_A-selective antagonist; Ref. 26), respectively, indicated the predominant expression of ET_B (Fig. 1b). From the biphasic displacement curve of ET-3 (whose affinity to ET_A is lower than that of ET-1), the ratio of the densities of ET_A and ET_B was roughly estimated to be 1:4 (Fig. 1b).

ET-1-induced Apoptosis of A375 Cells—Initial examination of the effect of ET-1 on A375 cell growth was done by measurement of the mitochondrial metabolism of the dye MTT which depends both on the number and viability of the cells. When applied to asynchronous cells in serum-free DMEM, ET-1 neither stimulates nor inhibits the cell growth within 24 h incubation (data not shown), but it caused a weak but significant inhibition of the FCS (1%)-dependent cell growth (Fig. 2a). In light of the cell cycle dependence of growth inhibition by somatostatin (16), we tested several synchronization procedures and found that the inhibitory effect of ET-1 was markedly enhanced when ET-1 was applied to the cells synchronized at G1/S boundary by double thymidine blocks (Fig. 2a). The growth inhibitory effect of ET-1 (5 nm) was completely blocked by BQ788 (1 μM) but not by BQ123 (1 μM) (Fig. 2a). Flow cytometric analysis revealed that ET-1 (5 nm) did not inhibit the FCS (1%)-dependent cell cycle progression after the release of the block up to 24 h, but it caused a significant increase of the hypodiploid cell peak (Fig. 2b) that is a characteristic feature of cells undergoing apoptosis (27). Time course analysis showed a 16-h time lag

---

FIG. 3. ET-1-induced chromatin condensation and DNA fragmentation. a, nuclear staining with PI. Phase-synchronized cells in glass-bottom dishes were incubated for 16 h in DMEM, 1% FCS with or without 5 nm ET-1. The cells were fixed and stained with PI, and fluorescent images were obtained by confocal microscopy. Arrowheads indicate cells with condensed chromatin. b, DNA fragmentation. Phase-synchronized cells were incubated for 16 h in DMEM, 1% FCS. The medium contained no drug (lane 1), 5 nm ET-1 (lane 2), 5 nm ET-1 with 1 μM BQ123 (lane 3), or BQ788 (lane 4). In separate experiments, the cells were treated with PTX (400 ng/ml) during the last 2 h of the second thymidine block and then were incubated for 24 h in DMEM, 1% FCS with 5 nm ET-1 (lane 5). The cellular DNA was extracted and separated on 1.2% agarose gel as described under “Experimental Procedures.” Molecular sizes are given on the right (in kilobase pairs). Shown are the representative results from three independent determinations.

FIG. 4. ET-1-induced cytoskeletal reorganization in A375 cells. Cells in poly-L-lysine-coated glass-bottom dishes were synchronized by double thymidine blocks and then incubated in DMEM, 1% FCS containing 5 nm ET-1. At 0 (a and d), 12 (b and e), or 24 (c and f) h incubation, the cells were fixed and double-stained with Texas Red-phalloidin and anti-a-tubulin antibody as described under “Experimental Procedures.” The anti-a-tubulin antibody was detected with biotinylated anti-mouse IgG and Cy2-avidin. In a control experiment, the cells were incubated for 24 h without ET-1 (g and h). Shown are the fluorescent images of Texas Red (a–c and g) and Cy2 (d–f and h) obtained by confocal microscopy.

---

2 M. Okazawa and H. Ninomiya, unpublished observations.
for ET-1 to cause a significant increase in the hypodiploid peak (Fig. 2c). ET-1-induced apoptosis was confirmed by the appearance of chromatin condensation on nuclear staining (Fig. 3a) and that of DNA fragmentation on gel electrophoresis (Fig. 3b). All of these effects of ET-1 (5 nM) were blocked by BQ788 (1 μM) but not by BQ123 (1 μM) (Figs. 2d and 3b). Treatment of the cells with PTX prior to the stimulation (400 ng/ml for 2 h) completely abolished the effect of ET-1 (Figs. 2d and 3b). In separate experiments using asynchronous cells, flow cytometric analysis failed to detect the hypodiploid peak regardless of the presence or absence of ET-1 (data not shown). Collectively, these results demonstrated that, when A375 cells were synchronized at G1/S boundary and then allowed to proceed the cell cycle, ET-1 inhibited the cell growth by inducing apoptosis and that the effect was transmitted by ETB via a PTX-sensitive G protein.

ET-1-induced Cytoskeletal Changes of the Surviving A375 Cells—Microscopic observation of the culture bed revealed that, when applied to the synchronized cells, ET-1 induced distinct morphological changes of the surviving cells. Within 12 h of stimulation with ET-1 (5 nM), more than 70% of the cells began to extend cytoplasmic processes that contained bundles of filamentous actin (stained with Texas Red-phalloidin) (Fig. 4b). After 24 h stimulation, the cells were apparently bipolar and elongated in shape and lost the bundles of filamentous actin (Fig. 4c). In contrast, a mesh-like organization of microtubules radiating from the centrosome in control cells (stained with anti-α-tubulin antibody) was apparently maintained in ET-1-stimulated cells (Fig. 4, d–f). These effects of ET-1 on the actin reorganization were blocked by BQ788 but not by BQ123 and were also blocked by pretreatment of the cells with PTX (data not shown). In separate experiments using asynchronous cells, less than 5% of the cells in

Fig. 5. ET-1-induced changes in p53 expression and localization. a, time-dependent changes in p53 level. Phase-synchronized cells were incubated for the times indicated in DMEM, 1% FCS with or without 5 nM ET-1. The cells were processed for immunostaining with anti-p53 antibody as described under “Experimental Procedures” and analyzed by flow cytometry. b, effects of ET receptor antagonists and PTX treatment. The cells were incubated for 16 h as in a with or without ET receptor antagonist as indicated. The concentration of ET-1 was 5 nM and that of BQ123/BQ788 was 1 μM. In separate experiments, the cells were treated with PTX (400 ng/ml) during the last 2 h of the second thymidine block and then incubated for 16 h in DMEM, 1% FCS with 5 nM ET-1. The cells were processed for immunostaining with anti-p53 antibody as described under “Experimental Procedures,” and fluorescent images of the cells were obtained by confocal microscopy. d, immunoprecipitation of metabolically labeled p53. During the last 7 h of the second thymidine block, the cells were metabolically labeled with [35S]Met/Cys. The cells were then incubated in DMEM, 1% FCS with or without 5 nM ET-1 for the times indicated. [35S]Met/Cys-labeled p53 was recovered from the cell lysate by immunoprecipitation and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. Molecular sizes are given on the right (kDa). Shown are the representative results from three independent determinations.
ET-induced Increase and Nuclear Accumulation of p53—Because of the well documented role of the tumor suppressor protein p53 in apoptotic cell death (28) and the somatostatin-induced increase of p53 in SST33-expressing cells (17), we examined the effect of ET-1 on the expression level and subcellular localization of this transcription factor in A375 cells. A375 cells have been reported to express only the wild-type p53 (29). When applied to the phase-synchronized cells, ET-1 caused a significant increase in p53 level as revealed by flow cytometry, and time course analysis showed that at least a 12 h exposure was required to cause the increase (Fig. 5a). The ET-1-induced increase was blocked by BQ788 and also by pretreatment of the cells with PTX (Fig. 5b). Immunocytochemical analysis revealed ET-1-induced nuclear accumulation of p53 (Fig. 5c) that was blocked by BQ788 and also by pretreatment of the cells with PTX (data not shown). Time course analysis showed that at least 6 h incubation was required for ET-1 to cause the translocation of p53 (data not shown). In an attempt to detect earlier changes in p53 metabolism, we conducted metabolic labeling and immunoprecipitation of the p53 protein. In accordance with the results of flow cytometry, there was no detectable increase in p53 protein level in ET-1-stimulated samples within 3 h incubation (Fig. 5d). After 2 h incubation, however, there appeared high molecular weight forms of the p53 most likely due to ubiquitination of the molecule (30, 31), and ET-1 caused apparent reduction of the high molecular weight forms (Fig. 5d). Despite repeated trials, we failed to demonstrate an increased survival of 35S-labeled p53 after a long term treatment with ET-1 (16 and 24 h), most likely due to the ET-1-induced apoptosis and hence the decrease of the total number of the cells in a well (data not shown). In separate experiments using asynchronous cells, ET-1 failed to affect the expression level and subcellular localization of p53 regardless of the presence or absence of FCS (data not shown).

Up-regulation of ETB Expressed in A375 Cells in G1/Early S Phases of the Cell Cycle—All of the effects of ET-1 on A375 cells described above were rather specific for the cells that were synchronized at G1/S boundary by double thymidine blocks and then allowed to undergo the cell cycle. In an attempt to explore the cell cycle-dependent modulation of ETB signaling, we examined the differences in receptor densities between the phases of the cell cycle. For this purpose, the receptor densities on individual cells were assessed by the binding of Cy2-labeled ET-1 and flow cytometry. Cy2-ET-1 binding capacities were highest in cells arrested at the G1/S boundary and gradually decreased as the cells underwent the cycle after the release of the block (Fig. 6a) to reach the level of those in asynchronous cells (not shown) at 24 h. The average Cy2 fluorescent intensities from the phase-synchronized cells were 5.2 ± 1.4-fold (mean ± S.E., n = 3) higher than those from asynchronous cells. This increase in Cy2-ET-1 binding capacities reflected an increase of ETB, because the binding to cell cycle-arrested cells was displaced by BQ788 but not by BQ123 (data not shown). To exclude the possibility that the increased ETB densities were due to the nature of double thymidine blocks, we used a plant amino acid mimosine as an alternative method of G1 phase synchronization (20). Mimosine treatment (0.1 mM for 18 h) of the log growth phase cells resulted in the late G1 phase synchronization as revealed by flow cytometric cell cycle analysis (data not shown), and as in the case of double thymidine blocks, the Cy2-ET-1 binding capacities were highest in the phase-synchronized cells (4.0 ± 2.7-fold increase, n = 3) and gradually decreased as the cells underwent the cycle after the removal of mimosine (Fig. 6b). These results indicated that the ETB densities on individual A375 cells were regulated by a cell cycle-dependent mechanism and that they were highest in cells in G1/early S phases of the cell cycle.

ET-1-induced Morphological Changes of A375 Cells Overexpressing ETB—To test the hypothesis that the increased ETB densities in G1/early S phases were responsible for the ET-1-induced responses, we microinjected an expression plasmid carrying human ETB cDNA (pEGFP/ETB) to asynchronous cells and examined whether the overexpression renders the cells responsive to ET-1. To identify the cells that were successfully injected and expressing the encoded proteins, we co-injected an expression plasmid carrying GFP cDNA (pEGFP). The relative ET-1 binding capacities of the cDNA-injected cells were estimated by Cy5-ET-1 binding assay followed by a densitometric analysis of the fluorescent images as described under “Experimental Procedures.” The Cy5-ET-1 binding capacities of pEGFP/pEhETB-injected cells were higher than those of control, pEGFP/vector-injected cells by 6.4 ± 1.0-fold (mean ± S.E., n = 50), being in a range similar to that observed in the phase-synchronized cells. In the pEGFP/pEhETB-injected cells, BQ788 (1 μM) almost completely inhibited the Cy5-ET-1 binding (0.7 ± 0.5-fold compared with the pEGFP/vector-injected cells, n = 30), whereas BQ123 (1 μM) caused only a very weak inhibitory effect (6.1 ± 1.0-fold, n = 30), excluding an obvious change in the expression level of ETBα in the ETB cDNA-injected cells. When the cells injected with pEGFP/pEhETBα were incubated in DMEM, 1% FCS in the presence of ET-1 (5 nM), a significant proportion of the GFP-positive cells (∼20%) shrank, and many of the shrunk cells just disappeared within 36 h incubation (Fig. 7, b and c). Also observed was elongation of >40% of the surviving GFP-positive cells (Fig. 7, b and c). In control experiments in which the cells were injected with pEGFP and empty vector cDNA, less than 5% of the GFP-
positive cells exhibited either shrinkage or elongation in response to ET-1 (Fig. 7c).

**Discussion**

When applied to A375 cells synchronized at G₁/S boundary, ET-1 did not inhibit the subsequent cell cycle progression but induced two phenotypical changes, apoptosis (Figs. 2 and 3) and cytoskeletal reorganization (Fig. 4). These phenotypical changes were preceded by ET-1-induced increase and nuclear accumulation of p53 (Fig. 5). All of these responses were rather specific for the synchronized cells. When applied to asynchronous cells, ET-1 caused neither apoptosis nor p53 increase/translocation, and ET-1-induced elongation was observed only in a fraction (<5%) of cells. Cell cycle synchronization by double thymidine blocks may be quite a stress to the cells that can predispose to apoptosis and/or cytoskeletal changes. However, neither the cell cycle synchronization per se nor the subsequent progression in 1% FCS caused increased expression/nuclear translocation of p53 that responds to a diverse repertoire of cellular stress. Indeed there was a portion of cells (<5%) with a hypodiploid peak (detected by flow cytometry; Fig. 2) when the cells were synchronized, but this was not accompanied with DNA fragmentation. Therefore, the apparent differences in the responsiveness of the cells to ET-1 between the synchronized and asynchronous states were not merely due to the synchronization-induced stress.

The relatively higher densities of ETβ expressed by the cells in G₁/early S phase (Fig. 6) and reproduction of the phenotypical responses by the receptor-overexpressing cells (Fig. 7) gave an alternative and the most likely explanation for the apparent differences that the synchronization-induced increase in the ETβ density was responsible for the phenotypical responses. The lack of responses in asynchronous cells is likely to reflect the fact that only a fraction of cells are in the G₁/early S phase and express relatively higher densities of ETβ. As for the control of the cell responses by receptor densities, it has been reported that PC12 or retinal progenitor cells overexpressing epidermal growth factor receptors underwent phenotypical differentiation when stimulated with the ligand that could not be induced at the normal level of receptor expression (32, 33). The present study described the first example of the apoptotic/cytoskeletal responses of the cells controlled by GPCR densities.
which, in turn, are regulated in a cell cycle-dependent manner. The molecular basis underlying the cell cycle-dependent regulation of ETβ expression is, however, present totally unknown and should be the subject of a future study. Also left unclarified was which signaling was augmented by the increased ETβ densities. In A375 cells, as in many other cell lines, ETβ activates phospholipase C to induce an increase in the intracellular calcium concentration ([Ca²⁺]i). However, we have failed to detect any difference between the [Ca²⁺]i responses of synchronized and asynchronous cells (data not shown). Therefore, it is unlikely that the augmentation of the classical phospholipase C/Ca²⁺ signaling caused the cell phase-specific apoptotic/cytoskeletal responses.

ETβ-mediated apoptosis of A375 cells was mediated by a PTX-sensitive G protein, namely Gi/o and Gq, and the present report constitutes the third example of apoptotic cell death induced by a GPCR via Gi/o, following those induced by ATI12 and SST3 (15, 17). In the literature, there is another example of apoptotic signaling mediated by Gi/o that is transmitted by amyloid precursor protein bearing mutations responsible for familial Alzheimer’s disease (35). Taken together, these studies indicate the presence of a Gi/o-dependent signaling pathway to apoptotic cell machinery. The molecular mechanism of the signaling pathway remains to be elucidated; however, studies on SST3R by Sharma et al. (17) and ours suggested the involvement of the transcription factor p53 in the signaling. In the present study, ETβ-induced reduction of the high molecular forms (most likely the ubiquitinated forms) of p53 preceded the increased expression and nuclear accumulation. Ubiquitin-dependent degradation is one of the major mechanisms to control cell cycle-associated proteins including p53 (36), and our results suggested the presence of the signaling pathway from G protein to the protein degradation system.

As in the case of the apoptotic signaling mediated by G proteins, the signaling pathways from GPCRs to cytoskeletal structures are still elusive, although several lines of evidence indicated an involvement of small GTPase proteins of the Rho family (37, 38). Because of the possible interaction of p53 with actin filaments (39, 40), we tested the effects of cytochalasin B on the ET-1-induced responses. Cytochalasin B, at the concentration (1 μM) high enough to block the ET-1 (5 nM)-induced actin reorganization, failed to block the p53 translocation and apoptotic cell death (data not shown). Therefore, the ETβ-mediated actin reorganization seemed rather independent from the apoptotic cell signaling. Pitzer et al. (40) described p53-mediated apoptosis of rat ovarian granulosa cells associated with actin reorganization and indicated the role of nuclear proteosomes translocated into the cytoplasm in the latter response. It remains to be determined whether the ETβ-mediated actin reorganization is due to the activation of signaling pathway that parallels the apoptotic signaling or is secondary to p53 mobilization.

Like neurons, melanocyte-precursor cells are derived from the neural crest, and it is well documented that apoptotic cell death is involved in the differentiation of various cell lineages, especially that of neuronal cells (18). ET-1-induced apoptosis of A375 cells was accompanied by morphological changes to the elongated, bipolar shape that apparently resembled that of MeWo cells that are supposed to be in a more differentiated stage than A375. However, ET-1 failed both to induce pigmentation and to alter the expression pattern of the marker antigens HLA-DR and gp100 (data not shown), suggesting that the differentiation stage was not altered by ET-1. Nonetheless, one may assume that the ET-1-induced phenotypical changes of A375 cells reflect at least part of the differentiation of melanocyte-precursor cells. Although the possible changes of receptor densities during the development of melanocyte lineage cells are not known, our results on the three melanoma cell lines suggested an increase through differentiation; the ETβ densities were in an order of H3K30 > MeWo > A375 that corresponds to the order of the relative differentiation stages from the highest. These results are in agreement with the decreased ETβ expression in metastatic melanomas (14) that are supposed to be in less differentiated stages than nonmetastatic melanomas. Together with the control of apoptotic/cytoskeletal responses to ET-1 by ETβ densities in A375 cells, these results suggested that the ETβ density may be a critical factor involved in the differentiation of melanocyte lineage cells.

In conclusion, we demonstrated the effects of ET-1 on A375 human melanoma cells that were mediated by ETβ coupled with a PTX-sensitive G protein. Both apoptotic and cytoskeletal responses of the cells were controlled by densities of ETβ expressed by individual cells which, in turn, were regulated by a cell cycle-dependent mechanism. These findings added a new dimension to the diverse biological activities of ETs and also indicated a novel mechanism to control the responsiveness of the cell to the peptides. The physiological relevance of the findings to the differentiation of melanocytes, however, remains speculative and will be the subject for future studies.

REFERENCES

ET-induced Apoptosis

Endothelin-induced Apoptosis of A375 Human Melanoma Cells
Makoto Okazawa, Takuma Shiraki, Haruaki Ninomiya, Shigeo Kobayashi and Tomoh Masaki

doi: 10.1074/jbc.273.20.12584

Access the most updated version of this article at http://www.jbc.org/content/273/20/12584

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 38 references, 9 of which can be accessed free at http://www.jbc.org/content/273/20/12584.full.html#ref-list-1