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HUMAN MELANOMA CELLS EXPRESS FUNCTIONAL ENDOTHELIN-1 RECEPTORS

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SUMMARY: Current evidence suggests that endothelium-derived factors enhance human melanoma vascular invasion. Therefore, we studied human melanoma cell expression of receptors to the endothelium-derived peptide, endothelin-1 (ET-1), and determined if they respond to ET-1 with proliferation and chemokinesis. Human metastatic melanoma cell lines were found to have specific, saturable, high affinity ET-1 binding. Northern analysis and competitive inhibition studies confirmed that melanoma cells express the ET_B receptor isoform. Ten nanomolar ET-1 caused an 8.2 to 25.5-fold increase in intracellular free calcium. ET-1 was found to be a weak mitogen for melanoma cells, however, melanoma cell chemokinesis was significantly increased by ET-1. These data suggest that ET-1 may be involved in providing a chemokinetic and growth factor environment that enhances perivascular proliferation and invasiveness of melanoma cells.

It has been recently demonstrated that normal human melanocytes (HM) express receptors for the bioactive peptide, endothelin-1 (ET-1) (1). ET-1 is equipotent to basic fibroblast growth factor (bFGF) as a melanocyte mitogen and ET-1 is co-mitogenic in an additive manner for HM with bFGF (1). Additionally, ET-1 has been shown to be a chemokinetic factor for normal HM (J.G. Morelli, unpublished data). Although ET-1 is secreted by human keratinocytes (2,3), human endothelial cells secrete over 100-fold more ET-1 than keratinocytes (3,4). Furthermore,

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ABBREVIATIONS:

bFGF: basic fibroblast growth factor; **ET**: endothelin; $[Ca^{2+}]_i$: intracellular free calcium.

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endothelial cells secrete ET-1 in an abluminal direction (5,6) allowing cells to react to ET-1 in the perivascular space.

ET-1 is secreted by mammary carcinoma, pancreatic carcinoma, and colon carcinoma (7). However, to date, there has been no melanoma cell line reported to secrete ET-1 (2,7). Cancer cell lines derived from human cervix and from human larynx express ET-1 receptors and respond to ET-1 with increased proliferation (8). Thus, ET-1 can act as a growth factor for some forms of cancer. It is possible that human melanoma cells may, like their precursor melanocytes, express ET-1 receptors and respond to ET-1 with enhanced proliferation and movement. Therefore, the purpose of these studies was to determine if human melanoma cell lines express specific ET-1 receptors and if ET-1 is a mitogenic and/or a chemokinetic factor for melanoma cells.

MATERIALS AND METHODS

Human Melanoma Cell Lines: Two human melanoma cells lines, Sloan Kettering Melanomas 28 and 30 (SK Mel 28 and SK Mel 30), were acquired from the American Type Culture Collection (Rockville, MD) and the third melanoma cell line, CU800 was acquired from the University of Colorado Cancer Center Tissue Culture Core. All cell lines were derived from metastatic lesions. Cells were maintained at 37°C, 5% CO_2, in RPMI 1640 (Gibco/BRL, Gaithersberg, MD) with 10% NuSerum (Collaborative Biomedical Products, Bedford, MA) and passaged 1:4 when confluent.

ET-1 Radioimmunoassay: ET-1 immunoreactive protein was measured in melanoma cell conditioned medium as previously described (3). Human umbilical vein endothelial cell conditioned medium was run as positive control. The ET-1 RIA utilized for these experiments can detect as little as 3.5 pg/tube ET-1 with less than 5% variability with duplicate tubes.

Preparation of Melanoma Cell Membranes: Cell lines were grown to confluence in 150 cm^2 flasks, six flasks per cell membrane preparation. For each cell line, the cells were washed with cold homogenization buffer (HB, pH 7.4 Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate buffered saline with 1.0 mM o-phenanthroline and 0.1 mM phenylmethylsulphonyl fluoride) and harvested by scraping into 5 ml ice cold HB. Cells were centrifuged at 4°C 400 x g for 10 min, washed with 5 ml cold HB and recentrifuged. Cells were resuspended in 3 ml cold HB and passed through a 20 gauge needle 5 times, then homogenized on ice in a Thomas A44545 cell homogenizer. The homogenate was then centrifuged at 4°C 100,000 x g for one hour, the supernatant discarded, and the crude membrane pellet resuspended in 4 ml HB. 50 ul of the membrane suspension was assayed for protein content by the method of Lowry (9)using bovine serum albumen as the protein standard.

Measurement of [^{125}I] ET-1 Binding: For ET-1 binding assays, 25 ul incubation buffer (50 mM Tris pH 7.4, 100 mM NaCl, 5 mM CaCl and 0.1% (w/v) sodium ascorbate) was added to 50 ul of the crude membrane prep (2.5 ug protein). 25 ul [^{125}I] ET-1 (New England Nuclear, Boston, MA) was then added to crude membrane prep aliquots to give a final concentration of 31.25 pM to 2 nM ET-1. Replicates also contained 1μM ET-1 (Peptides International, Louisville, KY) to determine non-specific binding. Binding was allowed to proceed for one hour at 4°C. Bound [^{125}I] ET-1 was separated from free by vacuum filtration through 2.4 cm diameter Whatman GF/B glass fiber filters (Whatman International Ltd, Maidstone, England) presoaked in 2% bovine serum albumin. Radioactivity trapped in the filters was measured with a Beckman Gamma 4000 gamma counter. Specific binding of [^{125}I] ET-1 was defined as total binding minus nonspecific binding in the presence of 1μM unlabeled ET-1. All assays were performed in duplicate and non-specific binding was approximately 30% of the total binding.

Measurement of Competitive Binding: Competition binding studies were carried out similar to above. [^{125}I] ET-1 (125 pM) in the presence of 10^{-11} M to 10^{-5} M unlabeled ET-1, BQ 123 (ET_A receptor-specific ligand) or sarafotoxin S6c (ET_B receptor-specific ligand) were used to determine receptor specificity. Binding, solubilization and measurement of membrane bound radioactivity was carried out as above. Binding in the presence of ET receptor-specific ligand was expressed as percent control (B/Bo x 100) of bound [^{125}I] ET-1.

RNA Isolation and Northern Analysis: Melanoma cell RNA isolation and northern analysis were performed as previously described (3). Human lung and passage 13 human umbilical vein endothelial cells were used as controls. The preproendothelin-1 probe is a (1.0 kb) full length human cDNA insert (4). The ET_A and ET_B receptor probes are full length (1.2 kb) human cDNA inserts isolated from human jejunum (10). The ß-actin probe is 104 base pairs from the untranslated region of human ß-actin kindly provided by L.H. Kedes (Stanford University School of Medicine, Palo Alto, CA).

Measurement of Intracellular Free Calcium Mobilization: 5 x 10^4 cells were plated onto 2.5 cm diameter round glass cover slips in 2 ml RPMI 1640/10% NuSerum and allowed to proliferate to 80% confluence. The cells were washed with phenol red-free M199 medium (Gibco/BRL) with 10% NuSerum and loaded with 3 µM Fura 2 AM plus 5 µM pluronic in phenol red-free M199/10% NuSerum at 37°C for 30 min. Following Fura 2 loading, the cells were maintained at 24°C to inhibit extrusion of the dye. Cells were washed twice with fresh M199/10% NuSerum and baseline 340 and 380nm signals were measured. The cells were then exposed to 10 nM ET-1 in M199/10% NuSerum by bath exchange and the 340/380 signals were measured every 10 seconds for 5 minutes. In vivo calibration for each cell type was obtained by measuring the Rmin and Rmax. Rmin was determined by measuring 340/380 signals following the addition of 10mM EGTA in 10 µM Ca^{2+} buffer and the Rmax was determined by measuring the 340/380 signals following the addition of 5 µM ionomycin in 100 uM Ca^{2+} buffer. There was no noticeable photobleaching of Fura 2 during the experiments. Intracellular free calcium concentrations ($[Ca^{2+}]_i$) were calculated from the following formula (11):

$$[Ca^{2+}]_i = [224]\ [R - Rmin/Rmax - R]\ [Fo/Fs] \qquad (1)$$

where Rmin = F_{340}/F_{380} without Ca^{2+}, Rmax = F_{340}/F_{380} at 100 µM Ca^{2+}, R = F_{340}/F_{380} at any experimental time point, and Fo = 380 signal without Ca2+ and Fs = 380 signal with Ca2+.

Measurement of Cell Proliferation: Each melanoma cell line was tested for growth response to 3 nM, 10 nM, or 30 nM ET-1. Cells were plated 5 x 10^3 cells per well in 6 well plates and cultured for 24 hours. This seeding density was chosen so that the melanoma cells would not be confluent on day seven of the experiments. Duplicate wells were used for each treatment group. Growth studies were performed on the cell lines in RPMI with 1) 10% NuSerum, 2) 3% NuSerum, 3) 10% NuSerum plus ET-1, or 4) 3% NuSerum plus ET-1. For each of the control and experimental conditions, culture medium was removed and the cells given fresh culture medium every 48 hours for seven days. On day seven, the culture medium was removed, the cells washed gently with phosphate buffered saline and counted by hemacytometer with trypan blue to confirm cell viability greater than 95%.

Specificity of the melanoma cell proliferative response to ET-1 was confirmed by anti-ET-1 antibodies. 1 x 10^5 cells per well were plated onto 6 well plates and cultured for 24 hours. The cells were then incubated in RPMI/3% NuSerum with 10 nM ET-1, 10 nM ET-1 plus 10 µg/ml purified rabbit anti-human ET-1 antibody (Peninsula Laboratories, Belmont, CA), or anti-ET-1 antibody alone for 20 hours followed by the addition of 1.0 µCi/well [^{3}H]-thymidine (35 Ci/mMole, ICN Biomedicals, Inc., Costa Mesa, CA) and incubation for 4 hours. Cells were then harvested, an aliquot counted by hemacytometer, and the remainder processed for scintillation counting by the method of Kragballe et al (12) with slight modification as previously described (13). [^{3}H]-thymidine incorporation was expressed as cpm/10^5 cells.

Measurement of Chemokinesis: Melanoma cell lines were cultured as described above. All experiments were performed using RPMI 1640/10% NuSerum. Time-lapse video microscopy was performed as previously described (14). Control and experimental flasks were viewed simultaneously by 2 separate time-lapse video microscopes utilizing a Nikon inverted microscope connected to a video camera and Javelin time-lapse video recorder. Temperature, humidity, and CO_2 concentrations were maintained at proper levels by an incubator hood enclosing the microscope stage. Total distance moved in 24 hours between the ET-1 treated, and control cells was compared by ANOVA utilizing SuperAnova software (Abacus Concepts, Berkeley, CA).

RESULTS

Immunoreactive ET-1 was not detected in the melanoma cell culture medium nor in undiluted NuSerum. Melanoma cell lines did not express prepro-ET-1 mRNA and did not secrete

ET-1 immunoreactive protein (data not shown). Melanoma cell crude membrane ET-1 binding studies revealed K_d's and B_{max}'s of 548 pM and 23 fmol/mg protein, 428 pM and 22 fmol/mg protein, and 247 pM and 13 fmol/mg protein for melanoma cell lines SKMel 28 and 30, and CU800, respectively (Fig.1). The number of ET-1 binding sites per cell was calculated to be 5.08 x 10^4 , 4.78 x 10^4, and 2.62 x 10^4 for SK Mel 28, SK Mel 30, and CU800 cell lines, respectively. As shown in Table 1, binding of [^{125}I] ET-1 was efficiently blocked with ET-1 and with the ET_B receptor-specific ligand, sarafotoxin S6C, but not with the ET_A receptor-specific ligand, BQ123.

All of the melanoma cell lines expressed ET_B (Figure 2), but not ET_A receptor mRNA (data not shown). Normal human lung expressed both ET_B receptor mRNA (Figure 2) and ET_A receptor mRNA (data not shown), whereas passage 13 cultured human umbilical vein endothelial cells expressed neither ET_A nor ET_B receptor mRNA as previously reported (10). Human lung had significantly lower beta actin expression compared to the melanoma cell lines.

The addition of 10 nM ET-1 to melanoma cell cultures caused an increase in $[Ca^{2+}]_i$ of 9.1-fold for SK MEl 28, 8.2-fold for SK Mel 30 and 25.5-fold for CU800 (Figure 3). The peak calcium response was followed by a slow decline in $[Ca^{2+}]_i$ over two to four minutes. The time interval for return to baseline $[Ca^{2+}]_i$ was similar for SK Mel 28 and SK Mel 30 cells whereas, the time interval was more than twice as long for the CU800 cells.

ET-1 stimulates enhanced proliferation of normal human melanocytes therefore, the effects of ET-1 on human melanoma cell proliferation were studied. Baseline cell growth with 10% and 3% NuSerum in RPMI was first determined. The population doubling time in RPMI/10% NuSerum was 1.11 days, 0.94 days, and 1.61 days for the SK Mel 28, SK Mel 30 and CU800 cell lines respectively. When 10 nM ET-1 was added to RPMI 1640/10% NuSerum, no significant increase in cell proliferation was noted for SK Mel 28 and SK Mel 30 cell lines whereas, the

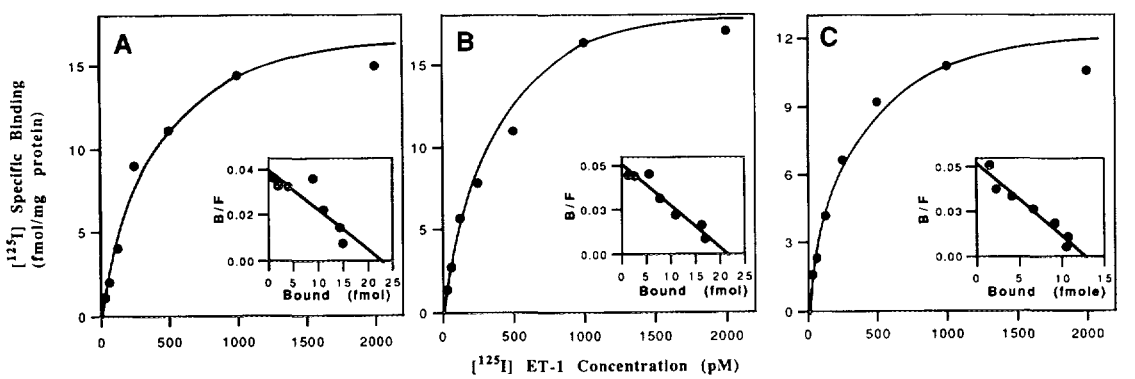

Figure 1.
The effect of [^{125}I]ET-1 concentration on specific binding to human melanoma cell membranes. Specific binding (fmol/mg protein) is shown for melanoma cell lines SK Mel 28, SK Mel 30, and CU800 (A, B, and C, respectively). Non-specific binding was approximately 30% of total binding. Insets: Scatchard plots of the concentration dependent specific binding data. Data are the means of duplicate determinations from two separate experiments.

Table 1

Competitive binding by ET-1, BQ-123 and sarafotoxin S6C in the presence of [125I] ET-1 to human melanoma cell membranes

	K_i[a]		
	ET-1	BQ-123	sarafotoxin S6C
SKMel 28	1 x 10^{-9}	2 x 10^{-5}	4 x 10^{-8}
SK Mel 30	1 x 10^{-9}	2 x 10^{-5}	1 x 10^{-7}
CU800	1 x 10^{-9}	2 x 10^{-5}	4x10^{-8}

K_i's were estimated from the percent of [125I] ET-1 bound relative to the amount bound in the presence of ET-1, BQ-123 or sarafotoxin S6C. Data are the means of duplicate determinations from two separate experiments.

[a] K_i represents the molar concentration of inhibitor that reduces [125I] ET-1 specific binding by 50%.

CU800 cell growth increased 123% ($P< 0.05$, Figure 4). For each cell line, 3% NuSerum in RPMI medium reduced cell growth by 50% compared to 10% NuSerum (Figure 4). When 3% NuSerum in RPMI was used, again the addition 10 nM ET-1 significantly increased the growth of CU800 cells (147% of control, $P< 0.05$). The growth of SK Mel 28 and Sk Mel 30 cells in 10 nM ET-1/3% NuSerum was increased to 118% and 120% of control, respectively, but these changes were not statistically significant (Figure 4). 3 nM and 30 nM ET-1 caused a 110% to 120% increase in cell growth for each cell line but the increase was not statistically significant ($P> 0.05$, data not shown).

The effect of ET-1 on melanoma cell [3H]-thymidine incorporation was also investigated. Exposure to 10nM ET-1 in RPMI medium containing 3% NuSerum significantly increased [3H]-thymidine incorporation ($P< 0.05$) in SK Mel 28, SK Mel 30, and CU800 cells to 127%, 140%, and 125% control, respectively (Figure 5). Anti-ET-1 antibody blocked ET-1 stimulation of melanoma cell [3H]-thymidine incorporation. However, anti-ET-1 antibody alone had no effect on melanoma cell [3H]-thymidine incorporation.

Figure 2.

Northern blot analysis of human melanoma cell lines. Equal amounts (20 µg) of total RNA were loaded, separated electrophoretically, and blotted. RNA was probed first with ET_A receptor cDNA. The membranes were stripped and rehybridized with ET_B receptor cDNA (2A), then stripped, and rehybridized with the human ß-actin probe (2B). Human lung and all three melanoma cell lines predominantly expressed the ≅ 2.2-kb ET_B receptor mRNA. However, human lung and melanoma cell lines SK Mel 30 and CU800 expressed ≅ 4.8-kb ET_B receptor mRNA. Passage 13 cultured human umbilical vein endothelial cells (HUVEC) did not express ET_B or ET_A receptor mRNA. The positions of 28S and 18S rRNA are shown at the left.

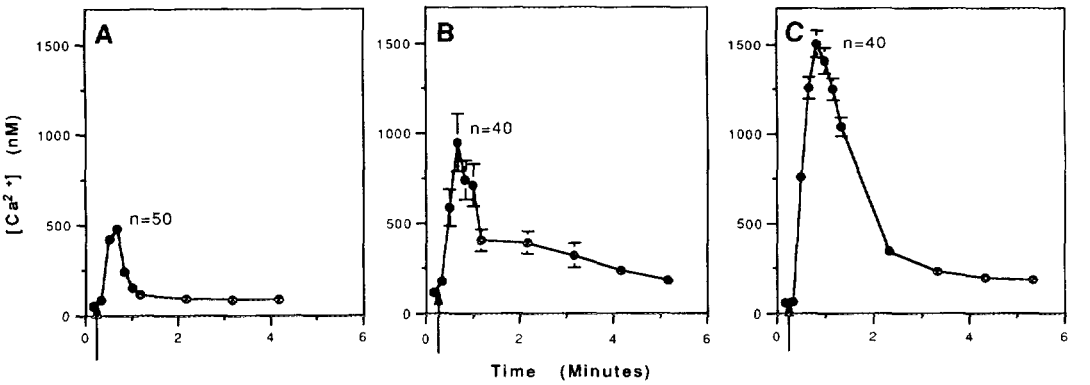

Figure 3.
The effect of 10 nM ET-1 on melanoma cell $[Ca^{2+}]_i$ mobilization. The data are expressed as the mean ± SE $[Ca^{2+}]_i$ of 40 to 50 Fura-2AM loaded cells for each cell line SK Mel 28 (3A), SK Mel 30 (3B), and CU800 (3C). ET-1 was added (arrow) immediately following a baseline $[Ca^{2+}]_i$ reading to provide a final ET-1 concentration of 10 nM.

Since ET-1 could act as a chemokinetic factor for human melanoma cells, the effects of 10 nM ET-1 on melanoma cell movement were studied. ET-1 caused statistically significant ($P < 0.05$) increased random movement (chemokinesis) of each of the three cell lines (Figure 6). In the

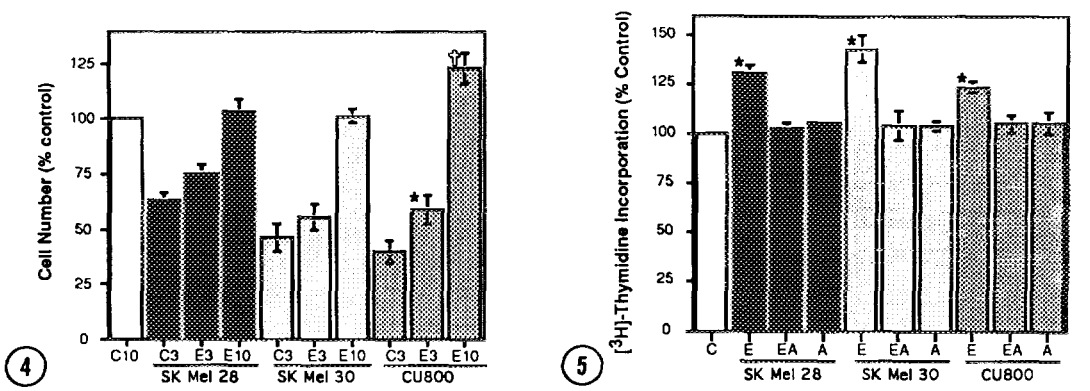

Figure 4.
The effect of NuSerum and ET-1 on melanoma cell proliferation. For each cell line, four cell culture conditions were evaluated: 1. RPMI 1640 plus 10% NuSerum (C10); 2. RPMI 1640 plus 3% NuSerum (C3); 3. RPMI 1640, 3% NuSerum plus 10 nM ET-1 (E3); 4. RPMI 1640, 10% NuSerum plus 10 nM ET-1 (E10). For each of the control and experimental conditions, culture medium was removed and the cells given fresh culture medium every 48 hours for seven days. Cells were harvested and counted by hemacytometer on day 7. Data are the means ± SE of duplicate determinations from three separate experiments. (*) signifies a statistically significant difference ($P < 0.05$) compared to 3% NuSerum control and (†)signifies a statistically significant difference ($P < 0.05$) compared to 10% NuSerum control.

Figure 5.
The effect of 10 nM ET-1 on human melanoma cell [^{3}H]-thymidine incorporation. All assays were performed in RPMI 1640/3% NuSerum. Cells were treated for 20 hours with either medium alone (C), 10 nM ET-1 (E), or 10 nM ET-1 plus 10 µg/ml anti-ET-1 antibody (EA), or 10 µg/ml anti-ET-1 antibody alone (A). The cells were then incubated with 1.0 µCi/ml [^{3}H]-thymidine for 4 hours and harvested, counted by hemacytometer and processed for scintillation counting. Data are the means ± SE of duplicate determinations from three separate experiments. (*) signifies a statistically significant difference ($P < 0.05$) compared to control.

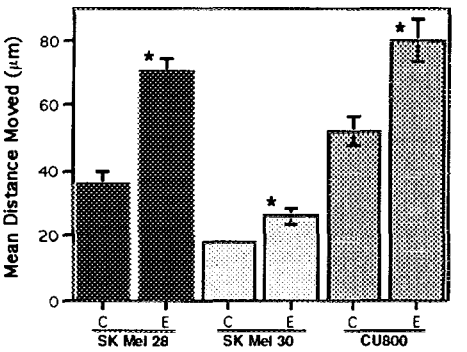

Figure 6.
The effect of 10 nM ET-1 on human melanoma cell chemokinesis. Melanoma cell lines were plated in RPMI 1640 plus 10% NuSerum with 10 nM ET-1 (E) or without ET-1 (C). Random cell movement was monitored by time-lapse video microscopy. Five separate experiments were performed for each cell type to acquire 24 to 33 cells in each group. Data are expressed as the mean ± SE. (*) signifies a statistically significant difference ($P < 0.05$) compared to control.

presence of 10 nM ET-1, SK Mel 30 cell movement increased to 144% control, CU800 increased to 160% control, and SK Mel 28 increased to 194% control.

DISCUSSION

These studies demonstrate for the first time the presence of active ET-1 receptors on human melanoma cells. All three melanoma cell lines express only the ET_B subtype as demonstrated both by competitive inhibitor studies and Northern analysis. Our results differ from those of Yada et al, who showed that HM express endothelin receptors of the ET_A subtype (1).

ET-1 receptors are a member of the seven transmembrane domain superfamily of receptors (15). These receptors are coupled to G-proteins that activate phospholipase C leading to the breakdown of membrane phospholipids, liberating two classes of second messengers, inositol phosphates and diacylglycerol. Inositol trisphosphate promotes the mobilization of calcium from intracellular stores (16), and inositol tetrakisphosphate has been implicated in translocation of extracellular calcium to the endoplasmic reticulum (17). Protein kinase C is activated by elevated levels of diacylglycerol at physiologic calcium levels (18). Melanoma cell ET receptor activation resulted in an 8.2 to 25.5-fold $[Ca^{2+}]_i$ increase. Although the magnitude of flux in melanoma cells is 2 to 6-fold higher than that reported for HM (1), it is similar in magnitude to the $[Ca^{2+}]_i$ response Savarese et. al. reported for melanoma cells binding type IV collagen (19).

Melanoma cells demonstrated only a nominal growth response to ET-1. This may be interpreted as loss of growth factor responsiveness by melanoma cells derived from metastases. It has been clearly demonstrated that as melanomas proceed to advanced stages of malignant progression, they acquire the ability to produce growth factors in an autocrine fashion and escape paracrine growth factor control of proliferation (20). Therefore, metastatic melanoma cell lines used in these studies may have lost paracrine growth response to ET-1.

Despite the modest proliferative response to ET-1, all the cell lines had a significant ET-1 chemokinetic response. Tumor cell motility is a critical requirement for invasion and metastasis (21,22) and tumor motility factors have been described for melanoma cells (23,24). Motility factors can enhance either random or directed movement (25) and do so either in an autocrine manner, such as autocrine motility factor (23), or in a paracrine manner, such as scatter factor (26). ET-1 appears to work in a paracrine fashion stimulating melanoma cell movement and may affect melanoma cells both in early primary tumors and in metastases. In early developing primary tumors, endothelial cell derived ET-1 may enhance tumor cell movement and may increase the invasive potential of these cells and possibly select out a clone of cells of higher invasive potential than the rest of the tumor. This hypothesis is consistent with the tumor progression scheme developed by I. J. Fidler wherein the process of invasion and metastasis consists of a series of successive, interrelated steps associated with the selection of clones with increasing invasive potential (27). In metastases, undergoing the transition from tumor embolus to an extravasating tumor (22), ET-1 may enhance tumor cell motility thus accelerating the extravasation process. Further studies on melanoma cells from early primary lesions, advanced primary lesions and metastases evaluating the effects of ET-1 on chemotaxis, integrin expression, protease secretion, and in vitro and in vivo invasion will help to better clarify the role of ET-1 in melanoma perivascular proliferation and invasion.

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