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ESTRADIOL AND FIBULIN-1 INHIBIT MOTILITY OF HUMAN OVARIAN- AND BREAST-CANCER CELLS INDUCED BY FIBRONECTIN

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Ovarian-cancer cells are characterized by their ability to invade freely the peritoneal cavity. Estradiol stimulates the proliferation of estrogen-receptor(ER)-positive ovarian-cancer cells, as well as expression of fibulin-1, a fibronectin-binding extracellular matrix protein. Using a modified Boyden-chamber assay, we have evaluated the respective roles of estradiol and fibulin-1 on cell motility, one of the earlier steps of tumor invasion. The effect of estradiol was examined on the random and directional migration of different ER-positive ovarian-cancer cell lines. The effect of fibulin-1 was studied on the motility of the MDA-MB231 breast-cancer cell line, which does not express fibulin-1. We found that when fibronectin (FN) was used as an attractant, estradiol decreased the cell motility of 2 ER-positive ovarian-cancer cell lines, BG-1 and SKOV3, but had no effect on 2 ER-negative cell lines, PEO14 and MDA-MB231. The inhibitory effect of estradiol was not observed when collagen (type 1 or 4) or laminin were used as attractants. Fibulin-1 was found to inhibit haptotactic migration of MDA-MB231 cells to FN in a dose-dependent manner. We conclude that both estradiol and fibulin-1 inhibit cancer cell motility *in vitro* and therefore have the potential to inhibit tumor invasion. *Int. J. Cancer* 75:654–658, 1998.
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Estrogens are known to stimulate the proliferation of hormone-dependent breast and ovarian cancers through their interaction on estrogen receptors and the expression of a number of genes, some of them involved in the control of cell proliferation (Dickson *et al.,* 1986; Vignon *et al.,* 1986). The effect of estrogen on invasion and metastasis has been much less studied, since the estrogen-receptor (ER)-positive breast-cancer cells are generally poorly invasive *in vivo* and *in vitro* as compared with ER-negative cancer cells (Price *et al.,* 1990). We have shown that the expression of fibulin-1, a FN-binding extracellular-matrix protein (Balbona *et al.,* 1992; Pan *et al.,* 1993) is markedly induced by estrogen treatment in 3 ER-positive ovarian-cancer cell lines (Clinton *et al.,* 1996; Galtier-Dereure *et al.,* 1992). While the structure of fibulin-1 (Argraves *et al.,* 1990; Pan *et al.,* 1993), its normal tissue distribution (Roark *et al.,* 1995; Spence *et al.,* 1992), and its ability to interact with different extracellular-matrix components (Balbona *et al.,* 1992; Pan *et al.,* 1993) are well documented, the function(s) of this protein is/are currently unknown, as is the biological significance of its estrogen-augmented secretion by ovarian epithelial cancer cells in culture. The expression of fibulin-1 has been shown to correlate with sites of active cellular migration in the embryo (Spence *et al.,* 1992). It has therefore been thought to play a role in regulation of cell migration.

The overall effect of estrogens on human ovarian cancer is debatable (Rao and Slotman, 1991). On the one hand, estrogen-replacement therapy has been suspected of increasing the risk of ovarian cancer (Rodriguez *et al.,* 1995), and the growth of ER-positive ovarian-cancer cells is stimulated by estradiol *in vitro* (Galtier-Dereure *et al.,* 1992; Langdon *et al.,* 1994). On the other hand, unlike breast cancers, ovarian cancers are generally resistant to anti-estrogen treatment, although they contain functional ER and ER-positive ovarian cancers may have a better prognosis than ER-negative cancers (review in Rao and Slotman, 1991). The poor prognosis of ovarian cancer is due to late diagnosis and to the ability of ovarian-cancer cells to invade the peritoneal cavity and to develop distant colonies. While this later step may be facilitated by mitogens such as estradiol, we have also considered that estrogen may have a role in the invasion process, which involves the migration of cancer cells in the presence of components of the extracellular matrix (Liotta *et al.,* 1991). We have therefore examined the role of estradiol and fibulin-1 in cancer cell motility by studying their effects on the *in vitro* migration of human ovarian- and breast-cancer cell lines.

MATERIAL AND METHODS

Proteins

Human plasma FN was purchased from Chemicon (Temecula, CA). Laminin, type-IV collagen, type-I collagen, gelatin and bovine serum albumin (BSA), were obtained from Sigma (St. Louis, MO). Human fibulin-1 was isolated from human placenta by immuno-affinity chromatography as described by Balbona *et al.* (1992).

Cell culture

Three ER-positive human ovarian-carcinoma cell lines, BG-1 (Geisinger *et al.,* 1989), SKOV3 (Hua *et al.,* 1995) and PEO4 (Langdon *et al.,* 1988), one ER-negative human ovarian-carcinoma cell line, PEO14 (Langdon *et al.,* 1988) and one ER-negative breast-cancer cell line MDA-MB231 (Cailleau *et al.,* 1974), were used. BG-1 and SKOV3 and MDA-MB231 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Cergy Pontoise, France) supplemented with 10% FCS (GIBCO). PEO4 and PEO14 were cultivated in RPMI-1640 supplemented with 10% FBS and 10 µg/ml insulin (Sigma). At 7-day intervals, the cells were passaged using trypsin-EDTA. Prior to estradiol treatment, steroids were removed by culturing cells for 5 days, in phenol-red-free DMEM or phenol-red-free RPMI-1640 containing 5% or 10% FCS pre-treated with dextran-coated activated charcoal (DCC) as described by Vignon *et al.* (1986). The cells were then treated with estradiol (Roussel-Uclaf, Romainville, France) in ethanol (final ethanol concentration 0.1%) for 24 hr. Control culture received the ethanol vehicle (0.1%) alone.

Cell motility assays

These were performed in a modified Boyden-chamber assay using a Transwell 2-chamber insert (6.5 mm diameter) separated by a polycarbonate filter of 8 µm pores (Costar, Cambridge, MA).

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Ovarian-cancer cells and MDA-MB231 cells cultured in phenol-red-free DMEM supplemented with 5% DCC-treated FCS were harvested with EDTA-trypsin and suspended for 1 hr in phenol-red-free medium containing 5% DCC-treated FCS to recover. The cells re-suspended in phenol-red-free medium were added in the upper compartment. After incubation at 37°C, the Transwell insert was removed, rinsed with Dulbecco's PBS (DPBS), fixed with methanol and stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Cells on the upper surface of the filter were removed by a cotton swab. The number of cells on the lower surface of the filter was counted by fluorescence microscopy under a high-power field (400×). Eight fields were counted in each of 3 different experiments. Results were expressed as mean number of migrating cells/field ± SD.

Random migration was assayed using 10% DCC-treated FCS in both compartments and a 24-hr migration period. FCS was replaced by 0.1% BSA when the effect of various extracellular-matrix proteins was assessed separately on random migration of BG-1 cells. Both surfaces of Transwell filters were coated with FN (50 µg/ml in DPBS), laminin (50 µg/ml in DPBS), type-I collagen (50 µg/ml in 0.1 M acetic acid), type-IV collagen (50 µg/ml in 0.1 M acetic acid) or gelatin (100 µg/ml in 0.16 M acetic acid). Transwells were washed with DPBS and dried, and BG-1 cells (5×10^5) suspended in phenol-red-free DME medium containing 0.1% BSA were added to the upper compartment of each Transwell. After 24 hr incubation at 37°C, the number of cells on the lower surface of the filter was counted. The optimal incubation time required to count a sufficient number of cells having migrated was 24 hr for BG-1 and other ovarian-cancer cells and 6 hr for MDA-MB231 cells, which were more mobile in the absence of FCS.

To measure directional migration (chemotaxis or haptotaxis), we also used 0.1% BSA instead of FCS. In the chemotactic assay, the Transwell filters were coated with 50 µg/ml type-IV collagen to enhance cell attachment. FN was diluted to 50 µg/ml in phenol-red-free DMEM containing 0.1% BSA, and added to the lower compartment of each Transwell. The upper compartment received BG-1 cells (5×10^5) suspended in phenol-red-free DME containing 0.1% BSA. The number of migrating cells was counted after 24 hr incubation at 37°C.

To measure haptotaxis of BG-1 cells to FN, the under surfaces of the filters were coated with 50 µl of FN solution (100 µg/ml) and air-dried overnight. The Transwells were washed with DPBS, dried and placed in a 24-well culture plate (Falcon, Dickinson Labware, Lincoln Park, NJ). BG-1 cells (5×10^5) suspended in phenol-red-free DME containing 0.1% BSA were added to the upper compartment of each Transwell. Estradiol (10^{-8} M) in ethanol or ethanol vehicle alone was present in the upper and the lower parts of the Transwells. The Transwells were incubated at 37°C for 24 hr before the migrating cells were counted.

To evaluate the effect of fibulin-1 on the haptotactic response of MDA-MB231 cells to FN, the lower surfaces of the filters were coated with a mixture of 2 µg FN and various doses of fibulin-1 or BSA, as described above. MDA-MB231 cells (5×10^5) were added to the upper chambers and allowed to migrate for only 6 hr at 37°C in the absence of serum.

RESULTS

Effect of estradiol on random migration of ovarian-cancer cell lines

In order to choose the most sensitive cell line for investigating the effect of estradiol and fibulin-1 on motility of ovarian-cancer cells, we first tested several estrogen-responsive ovarian-cancer cells in a random migration motility assay using a modified Boyden chamber in medium containing 10% DCC-treated FCS with or without estradiol (10^{-8} M). As shown in Figure 1, the different cell lines displayed varying abilities to migrate. High motility was observed with the more invasive MDA-MB231 breast-cancer and PEO14 ovarian-cancer cell lines; both lines are ER-negative. The 3 ER-positive ovarian-cancer cell lines tested displayed quite different motility. The most motile cells were the BG-1 cells. Approximately 15% of these cells had migrated through the filter in the absence of estradiol. The relative motility of 3 cell lines was consistent with their *in vitro* morphology (Fig. 2). PEO4 cells, which formed a polygonal epithelial monolayer with strong intercellular interactions, did not migrate. BG-1 and SKOV3 cells displayed a more mesenchymal morphology and were more motile. The inhibitory effect of estradiol on BG-1 cell motility was repeatedly observed in 6 different experiments for early passages of BG-1 cells (up to 15 passages). The same effect was observed with 2 nM estradiol concentration, which is consistent with an effect mediated by the estrogen receptor and with concentrations efficient for inducing fibulin-1 (Galtier-Dereure *et al.*, 1992). The anti-estrogen 4-OH-tamoxifen (concentrations from 1 nM to 1 µM), however, displayed no activity alone on BG-1 cell motility, in conditions where estradiol was inhibitory, indicating that in this assay the anti-estrogen was not a partial estrogen agonist (data not shown).

We next tested various ECM proteins, coated on both sides of the filter, for their ability to stimulate cell motility in the absence of FCS. Figure 3 shows that laminin had no effect; type-I collagen and type-IV collagen were partly active; and FN was the best stimulator of cell migration for the BG-1 cell line. The number of migrating cells on FN-coated filters was approximately 20-fold higher then that on noncoated filters. Estradiol treatment inhibited the migration of BG-1 cells cultured on FN, but had no effect on migration on type-IV collagen, type-I collagen, laminin (Fig. 3) or gelatin (not shown) substrata.

Effect of estradiol and fibulin-1 on haptotactic and chemotactic migration

Estradiol also inhibited the directional migration of BG-1 ovarian-cancer cells when FN was present only in the lower compartment of the Transwell. The degree of inhibition by estradiol was similar whether FN was present as a soluble chemoattractant in the lower chamber (Fig. 4*a*) or adsorbed on the lower surface of the filter in a haptotactic assay (Fig. 4*b*).

The estradiol-induced secreted protein fibulin-1 being a strong FN-interacting protein (Balbona *et al.*, 1992), we next evaluated the effect of fibulin-1 on cancer-cell motility. Since we found that BG-1 ovarian-cancer cells secrete fibulin-1 also in the absence of estradiol, we decided to use MDA-MB231 cells, which do not secrete fibulin-1 (data not shown), but are invasive in the Transwell assay. As shown in Figure 5, fibulin-1 inhibited MDA-MB231 cell migration toward FN in a dose-dependent manner, whereas a control protein BSA had no effect. The findings were similar when cells were allowed to migrate for 24 hr (data not shown).

DISCUSSION

Two important properties of cancer cells are their ability to invade surrounding tissues through a basement membrane (Liotta *et al.*, 1991) and their ability to form metastatic foci at distant sites (Chambers *et al.*, 1995), a process requiring tumor cells to detach from the ECM of the primary tumor and to migrate to other sites. It is supposed that increased cell motility facilitates invasion and metastasis of malignant tumors (Liotta *et al.*, 1991; Hay, 1990). In this study, we show that estradiol decreases invasion and motility of ER-positive ovarian-cancer cells and that fibulin-1, an estradiol-induced ECM protein, has similar inhibitory activity. Among the ER-positive ovarian-cancer cell lines that we examined, BG-1 cells showed the strongest ability to migrate. It is well known that the motility of several normal cells and tumor cells is regulated by ECM proteins such as FN, laminin and type-IV collagen, as well as by their breakdown products (Akiyama *et al.*, 1995). The migration of BG-1 cells was stimulated by immobilized FN, and by type-I and type-IV collagen, whereas laminin coating had no influence on migration. Estradiol treatment decreased the motility of BG-1 cells on immobilized FN, but the migration of BG-1 cells on immobilized laminin, type-I collagen or type-IV collagen was not affected

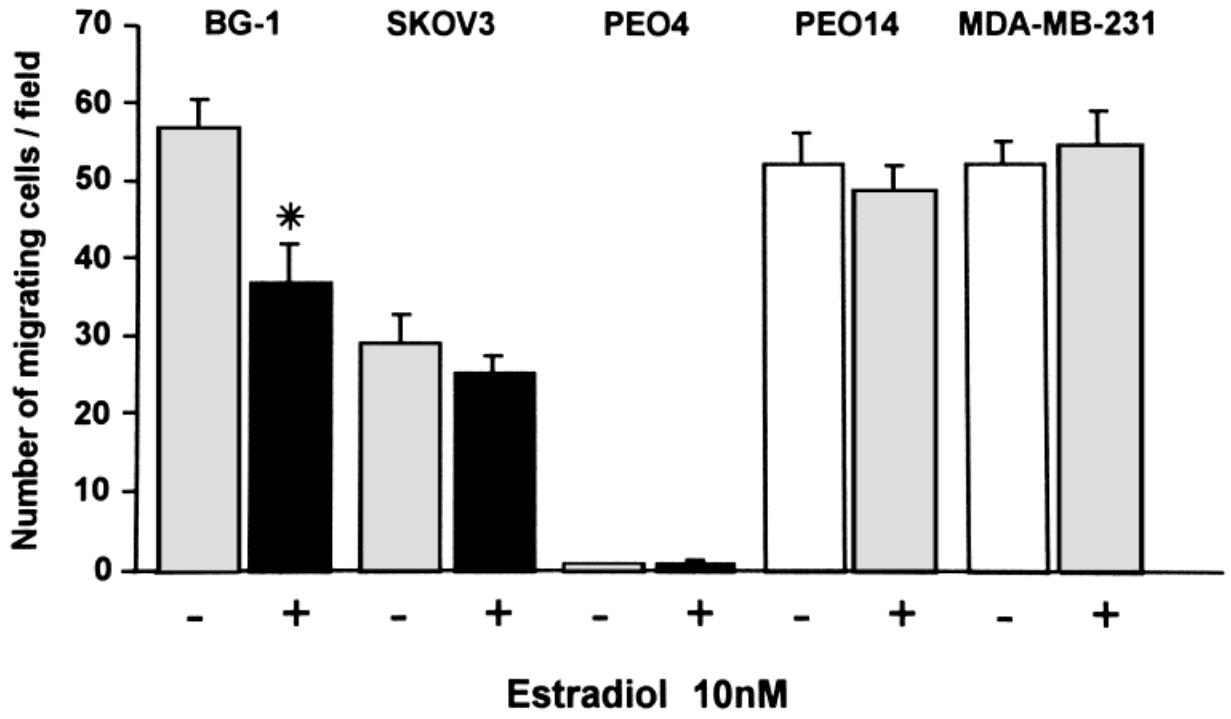

FIGURE 1 – Effect of estradiol on random migration of various cancer cell lines. BG-1, SKOV3 and MDA-MB231 cells (2×10^5) suspended in 200 µl phenol-red-free DME containing 10% FCS-DCC were added to the upper chamber. PE04 and PE014 cells cells (2×10^5) were suspended in phenol-red-free RPMI 164 containing 10% FCS-DCC. After 24 hr treatment with or without estradiol, the filters were removed, and fixed and the cell nuclei were stained by DAPI. The number of cells migrating to the lower surface of the filter was counted by fluorescence microscopy, as described in ''Material and Methods.'' Results derived from counting nuclei in 8 optical fields are given as mean ± SD of at least 3 different experiments **p* value < 0.01 according to Student's *t*-test. Other differences are not significant.

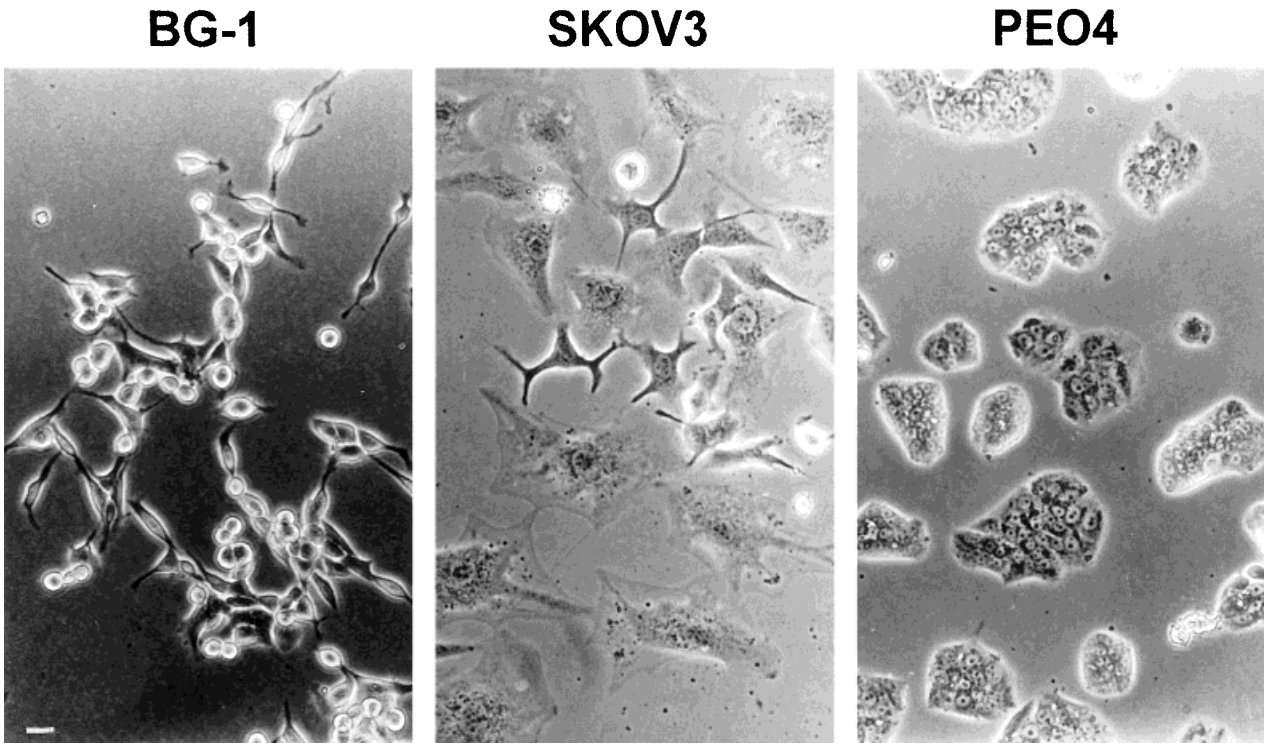

FIGURE 2 – Morphology of 3 estrogen-responsive human ovarian cancer cells. Cells were grown on plastic with 10% FCS, as described in ''Material and Methods'' and observed at low confluence by optical microscopy at the same magnification. Scale bar, 10 µm.

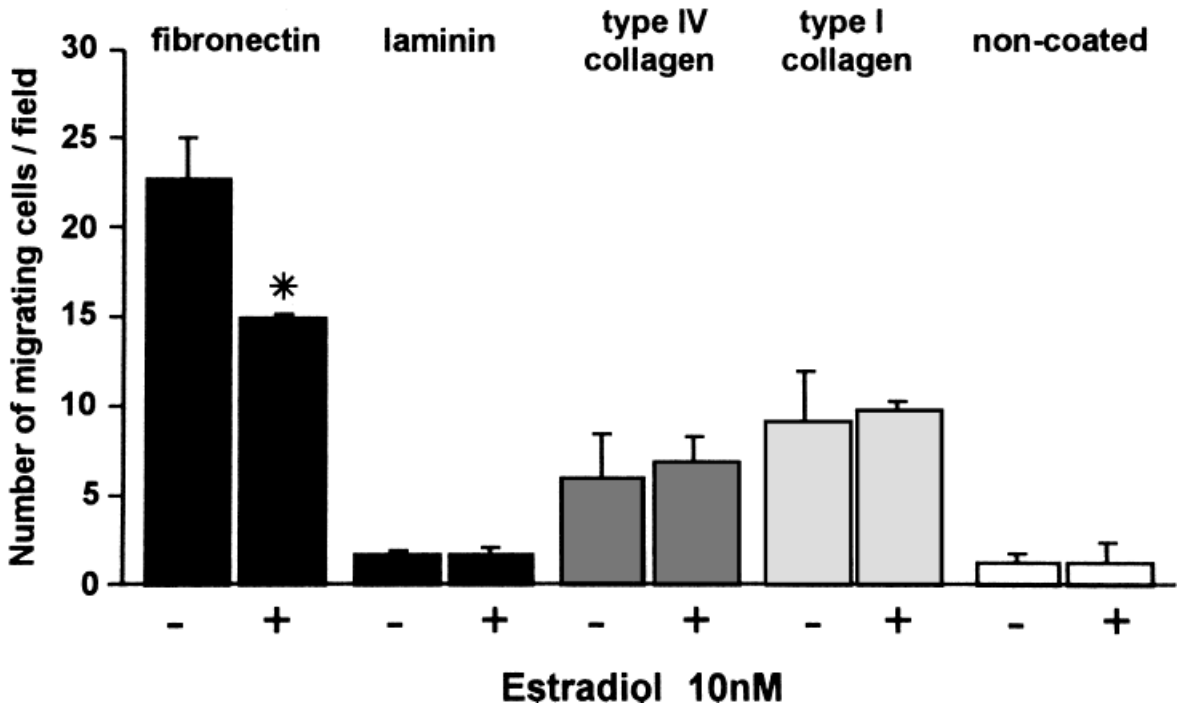

FIGURE 3 – Effect of estradiol on random migration of BG-1 cells on various ECM proteins. Transwell filters were coated by soaking in 50 µg/ml solution of the indicated ECM proteins. BG-1 cells (5×10^5) suspended in phenol-red-free DME containing 0.1% BSA plus or minus 10 nM estradiol were added to the upper chamber. After 24 hr treatment, cells were fixed and stained, and the number of cells migrating to the lower surface of the filter was counted. The results represent the mean number of cells per field ± SD. The effect of estradiol was seen only with fibronectin. *$p < 0.01$.

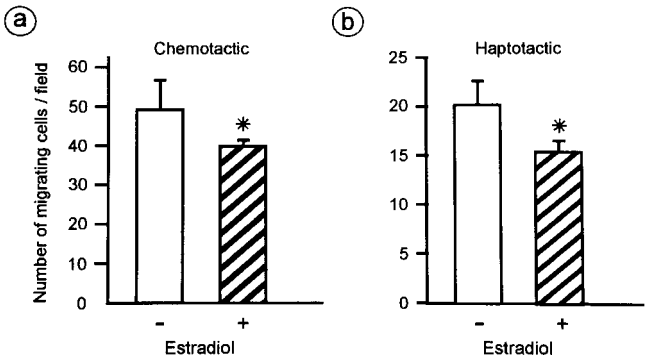

FIGURE 4 – Effect of estradiol on FN-induced migration of BG-1 cells. In the chemotactic assay *(a)*, 50 µg/ml FN was added to the lower chamber as a chemo-attractant. In the haptotactic assay *(b)*, the lower surface of the Transwell filter was dry-coated with 5 µg FN, as described in "Material and Methods." In both cases, BG-1 cells (5×10^5) suspended in phenol-red-free DME containing 0.1% BSA and plus or minus 10 nM E2 were added to the upper chamber. Following 24 hr incubation with or without E2 at 37°C, the cells on the lower surface of the filter were fixed, stained and counted as described before. The effect E2 was significant in both cases. *$p < 0.05$.

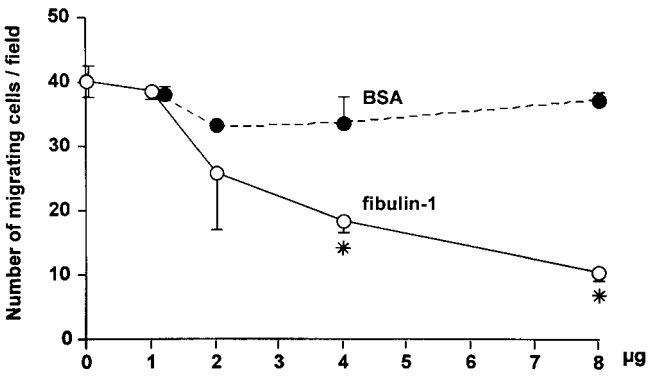

FIGURE 5 – Effect of fibulin-1 on haptotactic response of MDA-MB231 cells to FN. The lower surfaces of Transwell filters were coated with mixtures of 2 µg FN and the indicated amount of fibulin-1 or BSA. MDA-MB231 cells (5×10^5) suspended in phenol-red-free DME containing 0.1% BSA were added to the upper compartment. After incubation for 6 hr at 37°C, the filters were removed, and cells were fixed and stained, then counted in triplicate as described before. Result of one representative experiment is shown (mean ± SD). *$p < 0.01$.

by estradiol treatment. A similar inhibitory effect of estradiol on cell migration through Matrigel in a modified Boyden-chamber assay has been described in the ER-positive breast-cancer cell line MCF7 (Rochefort *et al.*, 1998) and in 4 clones established from two ER-negative cancer cell lines (MDA-MB231 and $3Y_1$-Ad12) stably transfected with ERα (Garcia *et al.*, 1992, 1996), suggesting that the inhibitory effect of estradiol on cell motility may be general as long as the cells contain the ERα.

The inhibition by estradiol of cell migration was initially surprising, since estrogens are known to be tumor promoters by facilitating the growth of ER-positive cancers. This suggests that a critical step in developing metastasis from a solid tumor may not be tumor-cell migration, but the ability of tumor cells to divide and form colonies in distant sites, as observed independently by intra-vital video microscopy (Chambers *et al.*, 1995).

We also show that fibulin-1 negatively affects FN-induced haptotactic cancer-cell migration *in vitro*. Since fibulin-1 has been

shown to interact with FN (Balbona *et al.,* 1992), we speculate that fibulin-1 may be able to inhibit the migration-promoting activity of FN. It is possible that the reduced cell migration on FN observed in estradiol-treated ER-positive ovarian-cancer cells was due to the blockade of FN-mediated cell migration by fibulin-1. The fact that fibulin-1 expression in the embryo was higher in a site of high cellular migration while fibulin-1 inhibits migration of cancer cells is noteworthy. It may not be contradictory if one assumes that fibulin-1 is over-expressed to control motility triggered by other factors. The mechanism of this inhibitory effect of fibulin-1 is not known, but is unlikely to be simply due to inhibition on FN-mediated cell attachment.

Similar interference with FN has been observed with tenascin (Chiquet-Ehrismann *et al.,* 1988). While fibulin-1 is known to be largely expressed by stromal cells and fibro-sarcoma, its high level of secretion by epithelial cancer cells may be characteristic of ovarian cancers, since we did not detect fibulin-1 secretion by breast-cancer cell lines (data not shown). Ovarian cancers, as compared with other solid tumors, are characterized by their free ability to invade the peritoneal cavity without necessarily breaking the basement membrane. In this respect, the control of cell motility and cell proliferation may be more important than that of invasion through a basement membrane to develop colonization at distant sites. In ovarian cancer, fibulin-1 may therefore serve as a mediator of estradiol action to decrease cell motility. However, this mechanism may not be general, since a similar inhibitory effect of estradiol on migration was observed in breast-cancer cells such as MCF7 and ERα-transfected MAD-MB231, which do not secrete detectable fibulin-1 (Garcia *et al.,* 1992, and 1996) and H Rochefort *et al.,* 1998). The general inhibitory effect of estrogens on cell motility observed in several ER-positive cells might therefore be due to multiple mechanisms, including other estrogen-regulated genes coding for proteins involved in the control of cell motility.

In conclusion, we report that treatment of cultured cancer cells with estradiol or fibulin-1 results in inhibition of their motility as tested both in random and in directional migration assays. The mechanism of these effects is not yet known. Their consequences may be important for understanding the action of estrogen and fibulin-1 on invasion and metastasis of solid tumor *in vivo* and in the emergence of new therapies.

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