Stromal fibroblasts from the interface zone of human breast carcinomas induce an epithelial–mesenchymal transition-like state in breast cancer cells in vitro

Ming-Qing Gao[1,2], Baek Gil Kim[1,2], Suki Kang[2], Yoon Pyo Choi[1,2], Hangran Park[1,2], Kyu Sub Kang[1,2] and Nam Hoon Cho[1,2,*]

[1]Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, 120-752, Korea
[2]Department of Pathology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemum-gu, Seoul, 120-752, Korea
*Author for correspondence (cho1988@yuhs.ac)

Accepted 1 July 2010
Journal of Cell Science 123, 3507-3514

doi:10.1242/jcs.072900

Summary

Fibroblasts were extracted from tissue in tumor burden zones, distal normal zones and interface zones between tumor and normal tissue of human breast carcinomas, and the corresponding fibroblasts were designated as cancer-associated fibroblasts (CAFs), normal zone fibroblasts (NFs) and interface zone fibroblasts (INFs). The crosstalk between three types of fibroblasts and breast cancer cells was evaluated using an in vitro direct co-culture model. We found that INFs grew faster and expressed higher levels of fibroblast activation protein than did NFs and CAFs. Compared with CAFs and NFs, INFs grown with breast cancer cells were significantly more effective in inducing an epithelial-mesenchymal transition (EMT) in cancer cells, as indicated by induction of vimentin and N-cadherin and downregulation of E-cadherin. This EMT process was also accompanied by activation of extracellular signal-regulated kinase (ERK) and modulation of membrane-type 1 matrix metalloproteinase (MT1-MMP) expression. Additionally, INFs promoted breast cell migration to a larger extent compared with NFs and CAFs. Taken together, these findings indicate that INFs isolated from the tumor interface zone exhibited more robust biological modulatory activity than did NFs and CAFs isolated from normal and tumor zones of the same tumor tissue, suggesting that the interface zone of the tumor represents a dynamic region vital to tumor progression.

Key words: Breast carcinoma, Epithelial-mesenchymal transition, Stromal fibroblasts, Tumor zones, Tumor microenvironment, Interface fibroblasts

Introduction

Breast cancer is a major health problem that affects the lives of millions of women worldwide each year. High-throughput genetic, epigenetic and gene-expression analyses have enhanced the understanding of the relationship of early neoplastic lesions to normal breast tissue, and distinct molecular alterations are observed in the tumor-stromal and myoepithelial cells during the transition from pre-invasive to invasive disease (Sgroi, 2010). Considering that most tumor burdens follow the activation of tumor microenvironment (TME) remodeling, the normal zone that is remote from the tumor load might yet be unaffected. Theoretically, remodeling of the TME could occur most rapidly in the interface zone, a unique functional and molecular region between the tumor invasion front and the normal zone (Kang et al., 2010). It has been reported that invasion markers in tumor tissues are most dynamic and active within the interface zone, where active cancer invasion or epithelial–mesenchymal transition (EMT) occurs (De Wever et al., 2008; Kang et al., 2010).

Accumulating clinical and experimental evidences reveal that tumor development is intimately related to the complex interactions of the carcinomas with several distinct stromal cell types that together create the microenvironment of the cancer cells (Bhowmick et al., 2004; Mueller and Fusenig, 2004). The fibroblast is one of the most crucial components of the tumor microenvironment, which promotes the growth and invasion of cancer cells through the involvement in angiogenesis (Orimo et al., 2005), EMT (Radisky et al., 2005), progressive genetic instability, deregulation of anti-tumor immune responses (Djouad et al., 2003), and synthesis, deposition and remodeling of the extracellular matrix (ECM) (Bhowmick et al., 2004). Such fibroblasts, known as cancer-associated fibroblasts (CAFs), when mixed with oncogene-expressing mammary epithelial cells, promote faster tumor growth than fibroblasts extracted from cancer-free breast tissues (Orimo et al., 2005; Shekhar et al., 2001). CAFs also differ from normal fibroblasts in phenotypic properties, the expression of growth factors and ECM molecule synthesis (Lebret et al., 2007). These findings suggest that CAFs possess biological properties and functions distinct from those of normal fibroblasts. However, the functional contributions of fibroblasts located in the interface zone between the tumor invasion front and the remote normal zone to cancer cells remain poorly understood.

In this report, we tested the hypothesis that fibroblasts isolated from tumor burden zone, interface zone and remote normal zone of breast tumor tissue contribute distinctive microenvironmental influences on breast cancer cells. We found that fibroblasts isolated from different tumor stromal zones differed with respect to their ability to induce EMT (measured as induction of vimentin and N-cadherin expression and suppression of E-cadherin expression), modulate expression of membrane-type 1 matrix-metalloproteinase (MT1-MMP), activate the ERK signaling cascade and promote cancer cell migration. Our results provide evidence for a direct correlation between the stromal zone of human mammary carcinomas from which fibroblasts were isolated and the ability of fibroblasts to promote breast cancer cell progression.

Results

Comparison of primary fibroblasts isolated from different zones of human breast tumor tissue

Breast cancer tissues were acquired from six patients by macroscopic dissection after the following geographic mapping: (1) real tumor burden, termed the tumor zone, located at the epicenter of the tumor tissue; (2) normal-like tissue located up to 5 mm from the invasive front of the tumor, termed the interface zone; and (3) distal normal tissue located at least 10 mm from the tumor margin, termed the normal zone (Fig. 1A). This concept is different from the traditional concept in which the tumor margin is defined by the edge of the tumor burden that faces the normal zone (Fig. 1B). For reproducible spatial identification, small invasive ductal carcinomas with an area less than 10 mm^2 were included. Larger tumors (i.e. area>10 mm^2), those with vague boundaries or cases other than infiltrating ductal carcinomas that were not otherwise specified (NOS) were excluded. Regardless of marginal clearance, all cases included in the present study were divided into a definite tumor burden zone, a clear normal fibrofatty region and an intervening fibrotic interface zone, all of which were examined microscopically. The intervening interface zone is characterized by a relatively more fibrotic zone with much less fatty tissue than the surrounding normal zone.

The fibroblasts from normal, interface and tumor zones of the tissue were designated NFs, INFs and CAFs, respectively. In the current study, a set of NF, INF and CAF populations were successfully isolated from a patient and each type of fibroblast possessed the basic fibroblast characteristics of an identical, long, spindle-shaped morphology, strong expression of fibroblastic marker vimentin and being negative for epithelial markers such as cytokeratin and cytokeratin 5 (Fig. 2A). Subsequent experiments were carried out using these cells passaged for 2–10 population doublings (PDs).

INFs cultured in vitro grew significantly faster than CAFs and NFs. This difference was confirmed using a Cell Counting Kit-8 (Dojindo, Rockville, MD). After seeding at the same density (2000 cells/well) in 96-well plates and culturing for 5 days, the number of INFs was increased relative to NFs and CAFs (Fig. 2B). However, the apoptosis rates of the three fibroblast types, as analyzed by FITC-Annexin V/PI staining and flow cytometry, were similar (Fig. 2C).

Additionally, fibroblast activation protein (FAP) was variably expressed in the isolated NFs, INFs and CAFs, with the highest expression in INFs and the lowest expression in NFs (Fig. 2D). A similar pattern was found in tissue sections from which the corresponding fibroblasts were derived (Fig. 2E). Furthermore, to assess the stability of FAP expression in fibroblasts cultured in vitro, its expression was investigated at 2, 5 and 10 PDs of NFs, INFs and CAFs. Each type of fibroblast can maintain the phenotypic characteristic of FAP expression up to 10 PDs (Fig. 2D).

INFs induce an EMT state in MCF7 cells in an in vitro co-culture model

To determine whether the fibroblasts isolated from normal, interface and tumor zones have different capacities to modulate tumor progression, we cultured NFs, INFs and CAFs directly with MCF7 breast cancer cells, allowing direct cell-cell interaction between cancer cells and fibroblasts. After co-culture, cancer cells and CMFDA-stained (green) fluorescent fibroblasts were readily separated by fluorescence-activated cell sorting (Fig. 3A), allowing breast cancer cells and fibroblasts to be individually assessed in follow-up experiments.

Downregulation of E-cadherin, a universal epithelial marker, is an early indication of EMT (Li et al., 2003; Zeisberg and Kalluri, 2004). A western blot analysis of E-cadherin expression in breast cancer cells after co-culture with fibroblasts showed that E-cadherin protein levels were decreased in MCF7 cells co-cultured with NFs, INFs and CAFs compared with those cultured alone. INFs exerted a more robust effect on this aspect of the EMT process than did NFs or CAFs (Fig. 3B, left). The observed decrease in E-cadherin coincided with the induction of N-cadherin and vimentin. The induction of vimentin expression in MCF7 cells co-cultured with fibroblasts was also confirmed by immunofluorescence staining. MCF7 cells co-cultured with INFs showed a much higher percentage of vimentin-positive cells than did those co-cultured with CAFs or NFs (Fig. 3C).

When the highly invasive MDA-MB-231 breast cancer line was used, E-cadherin was not detectable by western blotting in cancer cells cultured alone or co-cultured with fibroblasts. However, N-cadherin was faintly induced and vimentin was significantly upregulated in MDA-MB-231 cells, and the extent of these changes was greater in cells co-cultured with INFs than with NFs or CAFs (Fig. 3B, right).

MMPs and ERK are involved in the EMT process induced by co-culture

Cells that undergo EMT also undergo alterations in their interaction with the surrounding ECM through increased expression of matrix

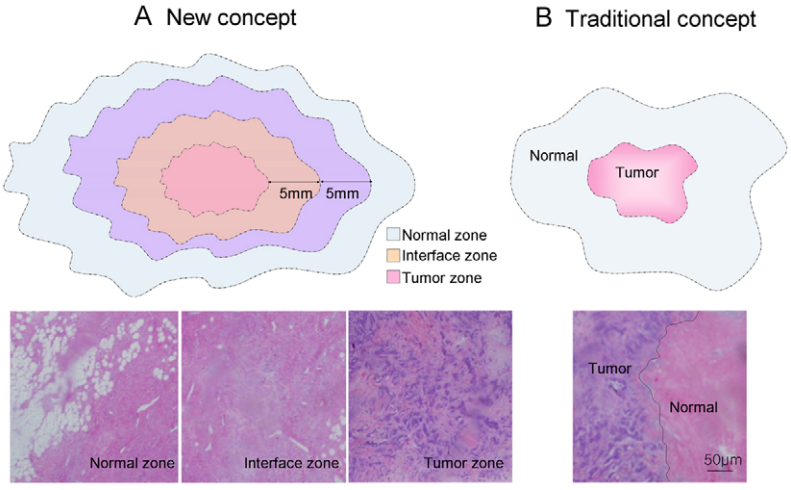

Fig. 1. Collection of representative breast tumor tissue samples. (**A**) Tumor tissue was grossly divided into the tumor zone, interface zone and normal zone as described in the text. The histological appearance was confirmed by hematoxylin and eosin (H&E) staining and a representative sample of tissue was collected from each region for subsequent fibroblast isolation. Microscopic findings showed a normal zone dominantly composed of intermingled fibrofatty tissue, an interface zone characterized by altered fibroplasias, and a tumor zone dominated by an invasive ductal type of carcinoma within the desmoplastic stromal response. (**B**) The traditional tumor margin was simply established as the edge of the tumor burden facing the normal zone.

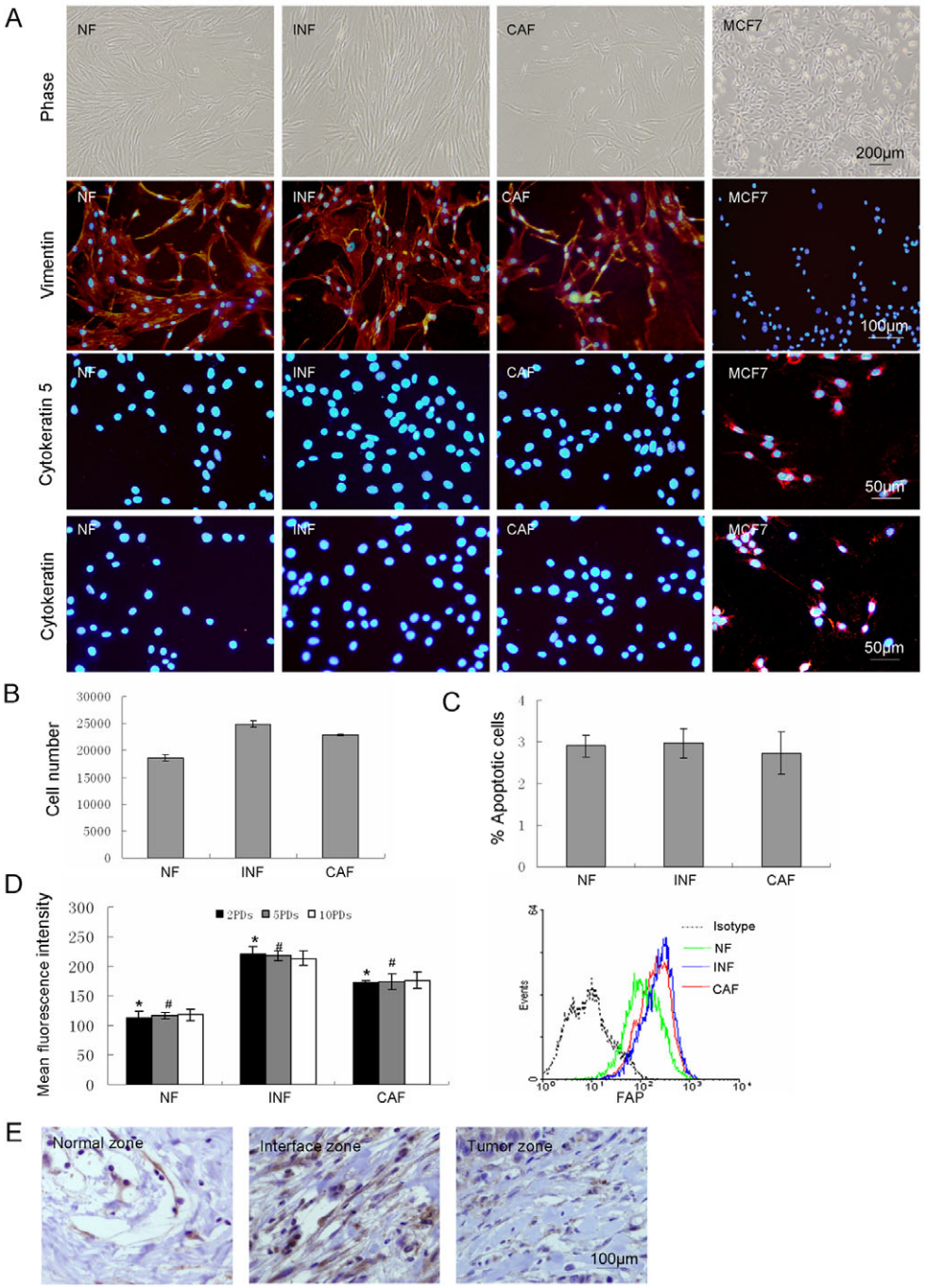

Fig. 2. Comparison of primary fibroblasts from each breast cancer tissue zone based on the new three-zone concept of tumor margins. (**A**) Normal zone fibroblasts (NFs), interface zone fibroblasts (INFs), cancer-associated fibroblasts (CAFs) and MCF7 cells in tissue sections were examined by phase-contrast microscopy and immunostaining for vimentin, cytokeratin 5 or a wide-spectrum cytokeratin; nuclei were stained with DAPI. The fibroblasts from each zone had an identical, long, spindle-shaped morphology and exhibited vimentin expression, whereas they were negative for cytokeratin 5 and cytokeatin. By contrast, MCF7 cells were negative for vimentin and positive for cytokeratin 5 and cytokeatin. (**B**) NFs, INFs and CAFs seeded at the same density (2000 cells/well) in 96-well plates were assayed for cell viability after 5 days in culture using Cell Counting Kit-8. Cell numbers were calculated by reference to a standard curve obtained under the same experimental conditions. Fibroblasts from the interface zone exhibited significantly greater growth rates compared with NFs and CAFs (P<0.01). The data were derived from three independent experiments. (**C**) NFs, INFs and CAFs grown to confluence were routinely collected and apoptosis was measured by Annexin/PI staining and flow cytometry analysis. The percentages of apoptotic cells were graphed and the data were derived from three independent experiments. A similar apoptosis rate was observed in each type of fibroblasts. (**D**) Fibroblast activation protein (FAP) expression was investigated by flow cytometry. A representative histogram of analysis and mean fluorescence intensity of FAP-positive cells in each type of fibroblast at 2, 5 or 10 population doublings (PDs) is shown. FAP was significantly overexpressed in INFs compared with NFs and CAFs at each PD (P<0.01); furthermore, for each fibroblast type, FAP was stably expressed at each PD (*P>0.05 versus 5 PDs and 10 PDs, $^{\#}P$>0.05 versus 10 PDs). The data were derived from three independent experiments. (**E**) Breast carcinoma sections were immunostained with anti-FAP antibody. FAP was stained specifically in fibroblasts, but this was only significant in the interface zone. Spindle cells are fibroblasts and the brown color indicates positive staining.

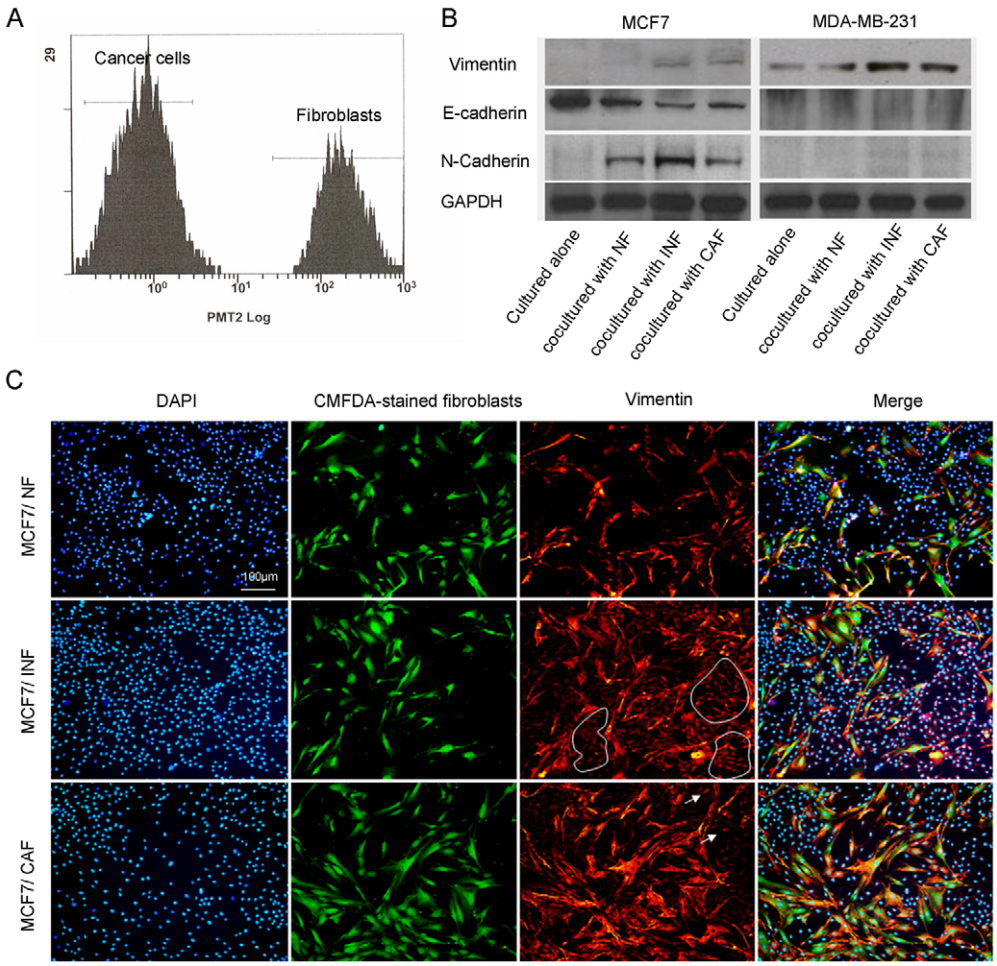

Fig. 3. INFs induced an EMT process in MCF7 cells in an in vitro co-culture model. (**A**) CMFDA-stained fibroblasts were distinguished from unstained cancer cells by flow cytometry. (**B**) Cadherin switching was most evident in MCF7 cells co-cultured with INFs. E-cadherin protein levels were decreased and N-cadherin levels were increased in MCF7 cells co-cultured with INFs to a greater extent than in those cultured alone or co-cultured with NFs or CAFs. E-cadherin and N-cadherin were undetectable in MDA-MB-231 cells, whether cultured alone or co-cultured with fibroblasts. Vimentin was highly expressed in both MCF7 and MDA-MB-231 cells co-cultured with INFs and CAFs. (**C**) Immunofluorescence staining for vimentin (red) in co-cultures of MCF7 and fibroblasts showed more frequent and stronger vimentin expression in MCF7 cells (indicated by polygon regions) co-cultured with INFs than in those co-cultured with CAFs (arrows) or NFs. Unstained MCF7 cells were distinguished from CMFDA-stained fibroblasts (green) in co-cultures under a fluorescence microscope; nuclei were stained with DAPI (blue).

MMPs (Orlichenko and Radisky, 2008). MCF7 cells cultured alone did not express MT1-MMP or MMP2, whereas both proteins were expressed in MDA-MB-231 cells, although the expression level of MT-MMP was faint. In MCF7 cells co-cultured in direct contact with primary tumor stroma-derived fibroblasts, INFs and CAFs significantly induced MT1-MMP expression, with INFs inducing higher levels of expression than CAFs. However, MMP2 expression was not induced in MCF7 cells by co-culture with fibroblasts from any stromal zone (Fig. 4A, left). Under the same co-culture conditions, both MT1-MMP and MMP2 were upregulated in MDA-MB-231 cells after co-culture with each of the three kinds of fibroblasts, with INFs showing a more robust capacity to upregulate expression than NFs or CAFs (Fig. 4A, right).

The RAF-MEK-ERK pathway confers epithelial cells with crucial motile and invasive capacities during development and carcinoma progression, often via promotion of EMT (Doehn et al., 2009). To determine if the ERK signaling pathway is activated during the co-culture-induced EMT process, we probed lysates of breast cancer cells co-cultured with different fibroblasts with antibodies directed against ERK and phospho-ERK. We found a greater degree of ERK activation, detectable as higher levels of phosphorylated ERK, in cancer cells co-cultured with INFs than in those co-cultured with NFs or CAFs (Fig. 4A).

The reciprocal influence of cancer cells on MMP2 induction in co-cultured fibroblasts was also examined by western blotting. Both MCF7 and MDA-MB-231 cells upregulated MMP2 expression in all three fibroblast types. Importantly, the degree of MMP2 upregulation was correlated with the malignancy of the tumor cell line: the levels of MMP2 were higher in fibroblasts co-cultured with the highly invasive MDA-MB-231 cells than in those co-cultured with the less-invasive MCF7 cells (Fig. 4B).

INFs promote cancer cell migration under co-culture conditions

Dissemination of cancer cells during the process of tumor metastasis, which is always enabled by EMT (Thiery, 2003), would

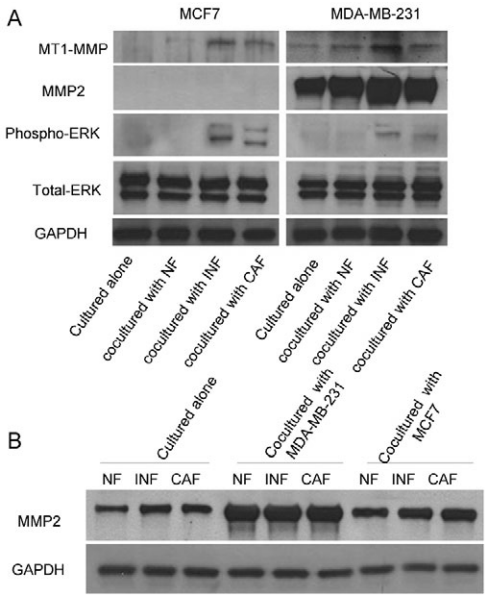

Fig. 4. Effect of interaction between fibroblasts and breast cancer cells on the expression of MT1-MMP, MMP2 and ERK in an in vitro co-culture system. After co-culture, both cancer cells and fibroblasts were isolated by FACS, which distinguished unstained MCF7 cells from the CMFDA-stained fibroblasts. Each cell type was also cultured alone as a control. (**A**) Expression of MT1-MMP, MMP2, total ERK and phospho-ERK in cancer cells. MT1-MMP was clearly induced in MCF7 cells co-cultured with INFs or CAFs, whereas MMP2 was undetectable in MCF7 cells cultured alone or with fibroblasts. Both MT1-MMP and MMP2 were induced in MDA-MB-231 cells co-cultured with any type of fibroblast. Note that MT1-MMP and MMP2 expression were most intense in MDA-MB-231 cells co-cultured with INFs. Both MCF7 and MDA-MB-231 cells co-cultured with INFs showed elevated levels of phosphorylated ERK. (**B**) MMP2 signals in fibroblasts co-cultured with the invasive MDA-MB-231 cancer cell line were more intense than those in fibroblasts co-cultured with the less-invasive MCF7 cell line.

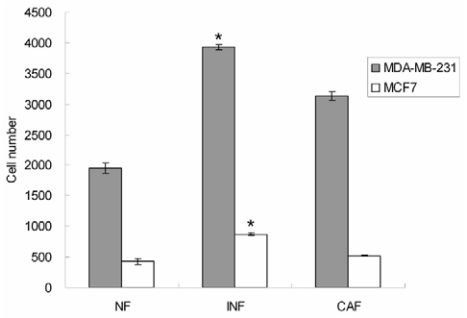

Fig. 5. INF-conditioned medium promoted migration of breast cancer cells. In vitro transwell chemotaxis assays were performed using conditioned medium from each fibroblast type. The number of either MCF7 cells or MDA-MB-213 cells that migrated toward the lower wells of the transwell containing particular fibroblast-conditioned medium was estimated using Cell Counting Kit-8. INF-conditioned medium significantly increased the number of both MCF7 and MDA-MB-231 cells that migrated toward lower wells of chambers compared with NFs and CAFs. *P<0.01 versus NFs and CAFs.

be expected to require enhanced migration ability. In light of the observation that INFs induced an EMT state in co-cultured MCF7 cells, we asked whether INFs promoted breast cancer cell migration. To examine this, we developed an in vitro transwell chemotaxis assay. We found a 2.1-fold and 1.7-fold increase in the number of MCF7 cells when INF-conditioned medium was used in the lower well of the chamber compared with NF- or CAF-conditioned medium. Similarly, 2.0-fold and 1.3-fold increases in the number of MDA-MB-231 cells migrated into lower wells of the chamber were observed when INF-conditioned medium was compared with NF- or CAF-conditioned medium (Fig. 5).

We also performed a scratch-recovery assay under direct co-culture conditions to investigate cell migration ability. After scratching, cells were allowed to recover and the capacity of cells to migrate to fill the area devoid of cells was assessed. When MCF7 cells were co-cultured with each type of fibroblasts, phase-contrast microscopic observations showed that the size of the scratched area decreased and nearly closed within 12 hours. However, fluorescent photomicrographs revealed that only in the co-cultures of MCF7-INF, and not MCF7-NF or MCF7-CAF, CMFDA-stained MCF7 cells (green) were observed to migrate into the scratched area (Fig. 6), indicating that, although all three types of fibroblasts migrated into the scratched area, only INFs were able to promote migratory behavior in the less-invasive MCF7 cells, although only to a low extent. Both phase-contrast microscopic observations and fluorescent photomicrographs showed that by 12 hours almost all MDA-MB-231-INF wounds were closed, whereas the wounds were still open in MDA-MB-231-NF or MDA-MB-231-CAF, although there was an obvious reduction of the wound width (Fig. 6).

Discussion

The results of the present study demonstrate potential differential interactions between fibroblasts from different tumor zones and breast cancer cells. Human breast CAFs and INFs grown with cancer cells dramatically promoted cell migration and induced an EMT process in cancer cells. This effect was not detected or was weaker when NFs were grown with cancer cells under the same experimental conditions. Importantly, INFs were more competent in promoting these changes in breast cancer cells than were CAFs. From these data, we conclude that INFs possess a greater capacity to interact with and modulate cancer cells in this human breast cell model system than do NFs or CAFs.

It should be noted that others have previously examined fibroblast and epithelial interactions in tumor growth and development by co-inoculating normal fibroblasts or CAFs with tumorigenic epithelial cells, showing that these fibroblasts stimulate mammary epithelial cell growth, differentiation and tumorigenesis or induce EMT via several possible pathways (Lebret et al., 2007; Orimo et al., 2005). Notably, the co-culture models used in these previous studies were indirect co-culture systems using transwells or conditional medium. Additionally, fibroblasts designated as normal in previous publications referred to fibroblasts isolated from human breast tissue from women undergoing reduction mammoplasty. By contrast, normal fibroblasts in the current study were defined as those isolated from the normal region of the same tumor tissue as CAFs and INFs were obtained from. In addition, we have define an interface zone that corresponds to a region located up to 5 mm from the invasive front of the tumor; this zone was grossly estimated by an experienced anatomical pathologist and confirmed by hematoxylin and eosin (H&E) staining. To the best of our knowledge, delineating normal, interface and tumor zones is a novel formulation that allows us to assess the direct

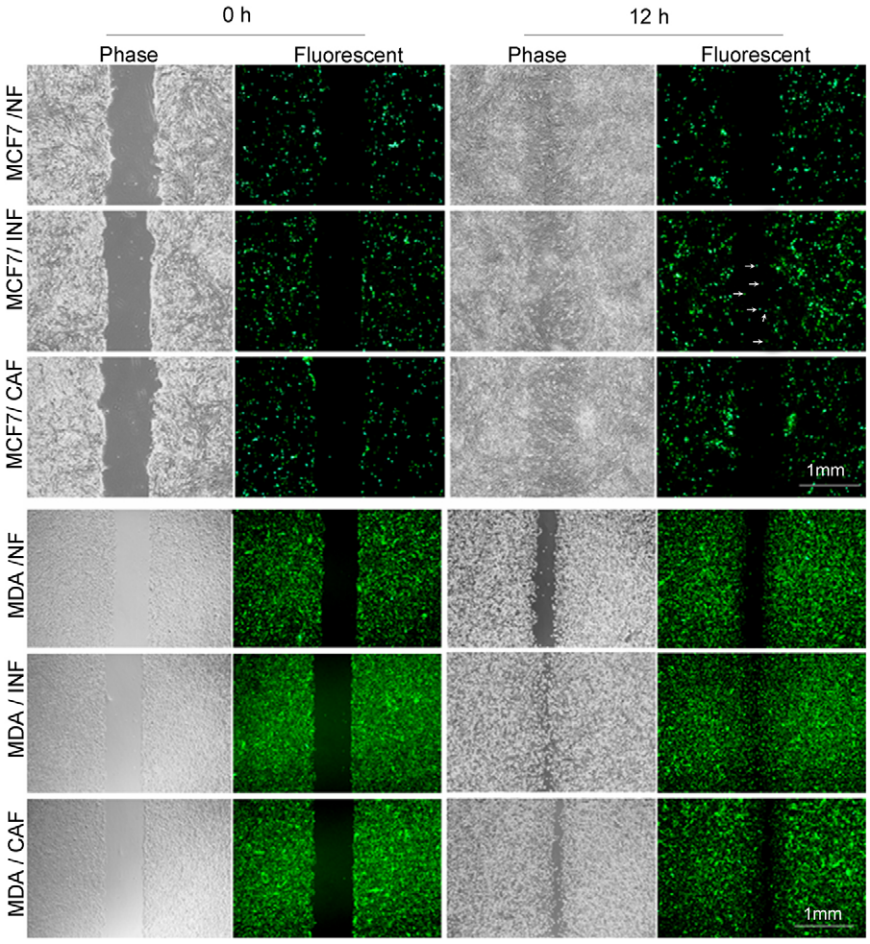

Fig. 6. Effect of fibroblasts on cancer cell migration in an in vitro direct co-culture system. The capacity of cells to migrate to fill a scratched area devoid of cells was assessed in co-cultures of cancer cells with NFs, INFs or CAFs. Before co-culture, breast cancer cells were pre-stained by CMFDA (green) to distinguish fibroblasts under a fluorescence microscope. For the co-cultures of MCF7 and each type of fibroblasts, phase-contrast microscopy showed that the size of the scratched area almost closed within 12 hours in all samples tested. However, fluorescence photomicrographs revealed that CMFDA-stained MCF7 cells only migrated into the scratched area of MCF7-INF (arrows) and never migrated into the scratched area of MCF7-CAF or MCF7-NF. For the co-cultures of MDA-MB-231 and fibroblasts from each stroma zone, by 12 hours MDA-MB-231-INF wounds were almost completely closed, whereas the wounds were still open in MDA-MB-231-NF or MDA-MB-231-CAF wounds in which there was also an obvious reduction of the wound width as indicated by both phase-contrast microscopy and fluorescence photomicrographs.

effects of the respective fibroblasts – NFs, INFs and CAFs – on EMT parameters and identify molecules involved in the EMT process.

Although NF, INF and CAF subpopulations were isolated from within the same specimen and exhibited identical, long, spindle-shaped morphologies, they could still be distinguished on the basis of differences in growth rate and FAP expression. The growth of solid carcinoma is partly determined by the balance between cell proliferation and apoptosis of its cells (Holmgren et al., 1995). Oda et al. suggested that both proliferation and apoptosis should be considered when predicting the growth of renal cell carcinoma (Oda et al., 2003). Our data showed that the increased number of INFs relative to NFs and CAFs on the basis of a cell-counting assay was not accompanied by either higher or lower apoptosis, indicating that this more rapid growth rate of INFs was mainly determined by the rapid proliferation rate. FAP is a cell-surface serine protease that is highly expressed in CAFs of human epithelial carcinomas but not in normal fibroblasts, normal tissues or cancer cells (Acharya et al., 2006; Park et al., 1999). A recent report also showed that FAP was detectable in normal mammary fibroblasts by western blot analysis (Lebret et al., 2007). Here, FAP expression was detectable in NFs, INFs and CAFs, with the highest levels observed in INFs.

A significant finding of this study is that MCF7 cells were susceptible to EMT transformations in the presence of INFs and CAFs, but not NFs, as indicated in part by the downregulation of E-cadherin and induction of N-cadherin. This ‘cadherin switching’, characterized by the loss of E-cadherin and the gain of N-cadherin, is a representative aspect of the EMT phenotype (Han et al., 1999; Tomita et al., 2000). The EMT marker vimentin was also induced by INFs and CAFs but not by NFs. In each case, these changes (downregulation of E-cadherin and upregulation of N-cadherin and vimentin) were much more strongly induced by INFs than CAFs. Additionally, although co-cultured NFs, INFs or CAFs had no effect on the expression of E-cadherin or N-cadherin in the highly invasive MDA-MB-231 breast cancer cell line, they did upregulate vimentin expression; similar to the observations in MCF7 cells, the effects of INFs were greater than those of CAFs and NFs. It is important to note that vimentin is not only an EMT marker but is also a myoepithelial-cell-specific protein; therefore, induction of this protein alone is not definitive for EMT. Because we do not mean to imply that MCF7 cells transform into fibroblasts, but rather assume a fibroblast-like phenotype, we have used the term ‘EMT-like state’ to describe the phenotypic changes induced by INFs and CAFs.

ERK stimulates most forms of epithelial invasive motility such as wound healing, EMT, malignant invasion and metastasis (Doehn et al., 2009; Horn et al., 2009). Our results showed that the EMT state induced by INFs and CAFs in MCF7 cells was accompanied by ERK activation. Although we showed that ERK was activated in the EMT process, we did not address the mechanisms by which ERK promotes the motility and invasive capacities of epithelial cells. However, the regulation of MMPs by activated ERK has been implicated in the invasive behavior of neuroblastoma cells

(Lakka et al., 2002) and it has been reported that MMP2 enzyme production is increased in fibroblasts cultured in the presence of media conditioned by MCF7 cells (Singer et al., 2002). In the present study, using a direct co-culture system, we provided evidence that breast cancer cells upregulated MMP2 expression in all fibroblasts (NFs, INFs and CAFs) and showed that the degree of MMP2 upregulation was positively correlated with the malignancy of the tumor cells. However, none of the three fibroblast types induced MMP2 expression in the less-invasive MCF7 cells, whereas each of these fibroblast types, but especially INFs, was capable of up-regulating MMP2 expression in highly invasive MDA-MD-231 cells. Interestingly, INFs upregulated the expression of MT1-MMP, the only activator of soluble MMP2 (Strongin, 2006) in both MCF7 and MDA-MB-231 cells. One possible reason that induction of MT1-MMP was not accompanied by MMP2 upregulation in MCF7 cells co-cultured with fibroblasts (except possibly indirectly by activating pro-MMP2 or other MMPs on the cell surface) is that MT1-MMP could directly degrade the ECM to create a path for cells to migrate (Itoh, 2006). Expression of MT1-MMP is reported to activate ERK, and ERK activation has been shown to be essential for MT1-MMP-dependent cell migration (Gingras et al., 2001; Takino et al., 2004); however, further work is needed to verify the mechanism by which MT1-MMP activates ERK.

As expected, INFs were able to promote migration of both MCF7 and MDA-MB-231 cells in a direct or indirect co-culture model to a larger extent when compared with NFs and CAFs, indicating that a factor, or factors, secreted or expressed by NFs and CAFs might be more highly secreted or expressed by INFs. Notably, the number of MDA-MB-231 cells that migrated towards lower wells of chambers was obviously higher than that of MCF7 cells, and INFs also enhanced the migration ability of MDA-MB-231 cells to a larger extent compared with the migration ability MCF7 obtained in a direct co-culture condition, which might be determined by their intrinsic properties. For instance, MCF7 is a less invasive cell line, whereas MDA-MB-231 is a highly invasive cell line.

In summary, our study demonstrated a direct role for INFs in breast cancer progression through the induction of an EMT state, the regulation of MMPs and ERK and promotion of cell migration. Compared with NFs and CAFs, INFs exhibited an increased propensity to induce the EMT program in MCF7 cells. Our study indicates that fibroblasts isolated from the interface zone of the tumor possessed more potent biomodulatory properties than NFs and CAFs isolated from normal and tumor zones, respectively. These results suggest that the interface region of the tumor, the site of EMT, represents a dynamic zone that is vital to tumor progression and local recurrence because of its robust biological activities.

Materials and Methods

Isolation of primary fibroblasts

Human breast tumor specimens were obtained from patients undergoing surgery at Severance Hospital of the Yonsei University Health System, Korea. An experienced anatomical pathologist grossly examined and obtained representative samples of tissue tumor from three zones: the tumor zone (tissue within the tumor boundary), an interface zone (adjacent tissue within 5 mm of the outer tumor boundary) and a normal zone (distal normal tissue at least 10 mm from the outer tumor boundary). A fraction of all tissues was fixed in formalin and embedded in paraffin for routine histopathological analysis. The remainder was used to isolate primary fibroblasts. In detail, fresh tissues obtained from different zones of the tumor tissue were cut into smaller pieces, placed in digestion solution of Enzyme Cocktail (ISU ABXIS, Seoul, Korea) and incubated at 37°C in a humidified 5% CO_2 incubator overnight. Digested tissue was filtered through a 70 μm cell strainer. The cells were suspended in medium:Ficoll (3:2) and separated by differential centrifugation at 90 ***g*** for 2 minutes. The supernatant containing fibroblasts was centrifuged at 485 ***g*** for 8 minutes, resuspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin with 100 μg/ml streptomycin (Gibco BRL, Grand island, NY) and cultured at 37°C in a humidified 5% CO_2 environment. The fibroblastic nature of the isolated cells was confirmed by microscopic determination of morphology portrait and immunofluorescence characterization using antibodies against vimentin (clone VI-10, diluted 1:1000, Abcam, Cambridge, UK), cytokeratin (clone AE/AE3, diluted 1:50, Dako, Glostrup, Denmark) and cytokeratin 5 (clone XM26, diluted 1:50, Novocastra, Newcastle upon Tyne, UK).

Direct co-culture experiments

Primary fibroblasts were directly co-cultured with human less-invasive MCF7 and highly invasive MDA-MB-231 (Korean Cell Line Bank, Seoul, Korea) breast carcinoma cells as described previously (Olumi et al., 1999). Briefly, adherent fibroblasts or breast cancer cells were stained by incubating for 45 minutes at 37°C in serum-free DMEM/F12 containing 5 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; Invitrogen, Eugene, OR), a green fluorescent dye. Subsequently, the dye solution was replaced with fresh, prewarmed medium and the cells were incubated for an additional 2 hours at 37°C. The cells were then washed twice with phosphate-buffered saline (PBS) and unstained cancer cells or fibroblasts were seeded onto plates containing CMFDA-stained fibroblasts or cancer cells, respectively. Finally, the co-cultures were incubated with minimum serum medium composed of DMEM/F12, 1% FBS and 100 IU/ml penicillin with 100 μg/ml streptomycin for 1 week. CMFDA-stained cells were easily distinguished from unstained cells by fluorescent microscopy and flow cytometry, allowing independent measurements of each cell type.

Immunofluorescence

Cells cultured in 8-well glass chambers (BD Biosciences, Bedford, MA) were rinsed twice with cold PBS, fixed in 4% paraformaldehyde for 20 minutes at room temperature and permeabilized with 0.2% Triton X-100 for 30 minutes on ice. Cells were then washed twice and incubated with 2% BSA in PBS for 1 hour at room temperature to reduce nonspecific binding of primary antibodies. Next, 500 μl of a primary antibody solution was added into each chamber and slides were incubated overnight at 4°C. After rinsing twice with PBS, cells were incubated with phycoerythrin (PE)-labeled secondary antibody for 1 hour at room temperature followed by subsequent washings. Finally, the glass slide was separated from the chamber and cells were stained with a drop of DAPI (Invitrogen) for 30 minutes to visualize nuclei and covered with a coverslip. Immunofluorescence was viewed using an immunofluorescence microscope.

Flow cytometry and fluorescence-activated cell sorting (FACS)

Detached cells were washed, resuspended in cold Hank's balanced salt solution (HBSS) containing 2% heat-inactivated FBS and blocked for 10 minutes with FcR reagent. Next, an anti-fibroblast activation protein (FAP) primary antibody (clone F11-24, Santa Cruz Biotechnology, Santa Cruz, CA) was added (1 μg per 10^6 cells) and incubated for 30 minutes on ice in the dark. Thereafter, cells were washed twice with PBS, incubated for 30 minutes on ice with PE-labeled secondary antibody and then analyzed on a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA). Cells in co-cultures were segregated using an EPICS ALTRA Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA).

Cell proliferation and apoptosis assay

Cell proliferation and apoptosis assays were performed using a Cell Counting Kit-8 (Dojindo, Rockville, MD) and FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA). For proliferation assay, cells were plated in 96-well plates and cultured in growth medium. At the indicated timepoints, the absorbance of samples in triplicate wells was measured with a VersaMax Microplate Reader at a wavelength of 450 nm and cell numbers were calculated based on reference to a standard curve obtained under the same experimental conditions. For apoptosis analysis, fibroblasts grown to confluence were routinely harvested and cells were then analyzed using the FITC Annexin V/PI Apoptosis Detection Kit according to the manufacturer's protocol.

Western blot

Cells were lysed in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea). Five to twenty micrograms of total protein from each sample was resolved on a NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) with MOPS running buffer and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were then probed with antibodies against GAPDH (clone V-18, diluted 1:2000, Santa Cruz Biotechnology), E-cadherin (clone H-108, diluted 1:200, Santa Cruz Biotechnology), vimentin (clone VI-10, diluted 1: 1000, Abcam), N-cadherin (clone H-63, diluted 1:1000, Santa Cruz Biotechnology), MMP2 (clone A-Gel VC2, diluted 1:1000, Thermo Fisher Scientific Anatomical Pathology, Fremont, CA), MT1-MMP (clone H-72, diluted 1:500, Santa Cruz Biotechnology), p44/42 MAPK (Erk1/2, diluted 1:1000, Cell Signaling Technology, Danvers, MA) or phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, diluted 1:1000, Cell Signaling Technology), followed by incubation with peroxidase-labeled secondary antibodies.

Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (Santa Cruz Biotechnology).

Wound healing assay

Breast cancer cells grown to approximately 50% confluence were stained with CMFDA and then co-cultured with fibroblasts, as described above. Cells were allowed to grow to 100% confluence and then simply wounded by making a single scratch in the monolayer with a pipette tip. The medium was then replaced to remove floating cells and debris and cells were incubated for 12 hours to allow cells to grow and close the wound. Photographs were taken at the same position of the wound.

Transwell chemotaxis assays in vitro

Confluent fibroblasts were rinsed in PBS and fresh serum-free DMEM/F12 medium was added to cell culture dishes. Cells were cultured for another 2 days at 37°C with 5% CO_2. Then medium was collected from the dishes and used at a 9:1 ratio in addition to fresh DMEM/F12 with 10% FBS as conditioned media after filtration in the lower well of a 24-well chamber (Costar, NY); 5×10^3 breast cancer cells were then seeded in 100 μl serum-free DMEM/F12 medium in the upper well of a Transwell chamber with 8 μm pore size filters. Transwell chambers were incubated for 1 week and the number of cells that migrated toward the lower chamber was estimated using Cell Counting Kit-8.

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 20090079165; No. 20090078398; CNH).

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