HUMAN PREADIPOCYTES INHIBIT PROLIFERATION OF MCF-7 BREAST CANCER CELL LINE

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Abstract- Cancer cell-stromal cell interaction plays a crucial role in the growth and invasion of tumor cells of various organs. It has been recently suggested that adipocytes and preadipocytes, two specific types of stromal cells, affect the biological behavior of variety of breast cancer cell lines. In spite of few investigations, there is a controversy in the literatures about the effects of adipocytes and preadipocytes on tumor growth. In this study, we compared the proliferation of MCF-7 breast-cancer cell line in culture with or without presence of primary isolated human adipocytes and preadipocytes by MTT colorimetric assay in 2, 4 and 6 days of culture experiments. Human adipocytes increased the proliferation of MCF-7 cells in the co-culture about 8.6%, 12% and 12% more than MCF-7 monoculture in 2, 4 and 6 days after cell culture respectively. Human preadipocytes decreased the proliferation of MCF-7 cells in the co-culture about 7.7%, 16.9% and 21% lesser than MCF-7 cell monoculture in 2, 4 and 6 days after cell culture, respectively. Proliferation of MCF-7 cells in co-culture with adipocytes were 17.7%, 35% and 42% more than MCF-7 cells in co-culture with preadipocytes after 2, 4 and 6 days of culture, respectively. We concluded that human adipocytes increase the proliferation of MCF-7 breast cancer cell line but human preadipocytes inhibit proliferation of MCF-7 breast cancer cell line.

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Key words: Co-culture, MCF-7 cell, subcutaneous human adipocyte, subcutaneous human preadipocyte, proliferation

INTRODUCTION

Breast cancer is the commonest malignancy in women and comprises 18% of all cancer (1).The past few years have provided substantial evidence for the vital role of the local environment of an emerging tumor for various steps of tumor genesis, including proliferation and local invasion.

Phenotypic behavior of malignant cells is regulated not only by cell autonomous signals, but also by effects exerted from surrounding stromal cells (2). In the breast, the main stromal cell types are fibroblasts and adipocytes. Fibroblasts are widely accepted as promoting the invasive growth of breast carcinoma cells (3). In fact, the majority of breast cancers have been referred to as “scirrhous” because

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of their extremely hard consistency provided by large numbers of fibroblasts dispersed between malignant epithelial cells, as well as within the immediate periphery of tumors. The relationship between adipose stroma and breast cancer is unique in the sense that stromal fibroblasts seem to provide the structural support for cancer growth, whereas malignant cells greatly influence the composition of the adjacent tissue. Evidence from several laboratories indicates that this epithelial-stromal interaction also involves paracrine mechanisms that promote the development and growth of breast carcinomas (4-6). These morphologically identified intra- and peritumoral fibroblasts originate from adipose tissue and most likely represent potential preadipocytes, because fibroblasts isolated from adipose tissue are capable of differentiating to mature adipocytes under defined culture conditions (7, 8). Although the suggestion exists that fibroblasts maybe originated from preadipocytes, but very little attention has been given to the adipocytes and preadipocytes, arguably the most abundant stromal cells in the breast (9). One of the reasons why the adipocytes received relatively little attention was the view that it is merely an energy-storing cell, and other reason is the difficulty in culturing mature adipocytes (1, 9). Work by many different laboratories over the course of the past few years has, in fact, shown that the adipocyte is a highly active endocrine cell that secretes numerous factors, including growth factors, cytokines, hormone-like molecules, acute phase reactants, complement-related proteins, and extra cellular matrix proteins (10, 11). The adipocytes and preadipocytes represent the most abundant cell types in the adipose tissue. In the adipose tissue, adipocytes and preadipocytes exist as mature cells and immature cells, respectively. The former constitutes most of the adipose tissue and has a large lipid droplet in the cytoplasm. The latter is fibroblast-like and has minute lipid droplets (12). Each of them differentiated and dedifferentiated to each other during puberty, pregnancy, post pregnancy and menopausal periods (13). In spite of few investigations, there is a controversy in the literatures about the effects of adipocyte and preadipocytes on tumor growth. We hypothesized that subcutaneous human adipocytes and preadipocytes as stromal cells and as environmental factors might play crucial roles on the behavior (proliferation and morphology) of breast cancer cell line. In this study, we compared the proliferation and morphology of MCF-7 breast-cancer cell line in culture with or without presence of human adipocytes and preadipocytes.

MATERIALS AND METHODS

Cell line and preparation of adipocytes and preadipocytes

As typical breast carcinoma cells types, we used MCF-7 (NCBI C135) breast cancer cell line (obtained from National Cell Bank of Iran).Human adipose tissue was obtained from the abdominal subcutaneous region (epigastric region of the abdominal wall) from elective or laparoscopic abdominal surgery (hernia, gall stone, and so on) performed at Imam Khomeini hospital. Except for obesity and minor metabolic disturbances, the subjects were healthy and took no regular medication. Informed consent was obtained from the subjects before the surgical procedure. After being removed, adipose tissue samples of 5 to 20 g were immediately transferred under sterile conditions to the laboratory. The subcutaneous adipose tissue specimens were dissected from fibrous material and visible blood vessels, minced into small pieces, and digested in phosphate-buffered saline (PBS) containing 20 mg/ml bovine serum albumin (BSA) and 250U/mL collagenase for 90 minutes at 37°C. The completely disaggregated tissue was centrifuged for 10 minutes at 200 × g; thereafter the supernatant containing mature adipocytes and collagenase solution was removed from the pellet that contains the preadipocytes. The pellet of preadipocytes was resuspended in an erythrocyte lysis buffer (consisting of 0.154 mol/L NH_4Cl, 5.7 mmol/L K_2HPO_4, and 0.1 mmol/L EDTA, pH 7.3) to remove contaminating red blood cells, filtered through a polypropylene mesh (pore size 150 μm) and centrifuged for 10 minutes at 200 × *g*. The cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, filtered

again (pore size 70 µm), and the total cell number was determined microscopically using a Neubauer chamber (14).

Primary culture of preadipocytes

The preadipocytes were plated in 25-cm^2 tissue culture flasks containing DMEM–Ham's F-12 medium (vol/vol, 1:1) supplemented with 10% fetal bovine serum, 15 mmol/L HEPES (pH 7.4), 100 U/ml penicillin, 100 µg/ml streptomycin. The initial plating density was approximately 5000 cells/cm^2. The primary cells were cultured for 4 to 5 days until they reached confluence (14). These preadipocytes expressed S-100 protein, which is a marker of preadipocytes (Fig. 1), but not of fibroblasts or endothelial cells (15).

Primary culture of adipocytes

The adipocytes were plated in 25-cm^2 tissue culture flasks containing DMEM–Ham's F-12 medium (vol/vol, 1:1) supplemented with 10% fetal bovine serum, 15 mmol/L HEPES (pH 7.4),100 U/ml penicillin, 100 µg/ml streptomycin. The initial plating density was approximately 5000 cells/cm^2. The primary cells were cultured for 10 days until they reached confluence (14).These adipocytes were stained by Oil red O (Fig. 2).

Adipocytes and preadipocytes growth arrest

Adipocytes and preadipocytes used in co-culture were arrested by treating with 20 µg/ml mitomycin C for 2 h and then were washed three times by DMEM–Ham's F-12 medium (vol/vol, 1:1)(16, 17).

Three-dimensional collagen gel culture system

We cultured 1×10^4 MCF-7 breast cancer cell together with or without 1×10^5 mitomycin C treated mature adipocytes or preadipocytes in ninety µl of three-dimensional collagen gel matrix. We analyzed the results obtained from materials cultured for 6 days. The collagen gel culture system was prepared as follows. Briefly, 8 volumes of type I collagen (Sigma) was mixed with 1 volume of ten-fold concentrated Ham's F-12 medium and 1 volume of a reconstruction buffer (2.2 g of $NaHCO_3$ and 4.77 g of HEPES in 100 ml of 0.05 N NaOH). Ninety µl of this collagen gel solution was mixed with the 1×10^5 mitomycin C treated adipocytes or preadipocytes as a basal cells culture. In co-culture experiments, 1×10^4 MCF-7 breast cancer cell was added to the collagen solution that contain basal cells. In monoculture experiments, 1×10^4 MCF-7 breast cancer cell was added to the ninety µl of collagen solution alone (control group). Ninety µl of collagen gel solution containing cells (co-culture or monoculture) was poured into a well of a 24-well cell culture plate. The culture plate was immediately warmed to 37 °C to allow a gel to form. The gel was further covered with 400 µl per well Ham's F-12 medium supplemented with 15% newborn calf serum and 50 µg/ml gentamicin and be kept for 24 h (day 0). After washing three times with PBS, they were cultured for 6 days. Medium was changed every second day (18).

Cell proliferation assay

Cell proliferation was measured by MTT

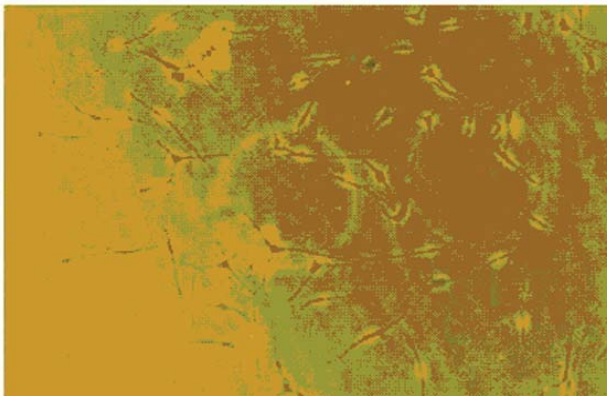

Fig. 1. Preadipocytes expressed S100-protein (magnification × 100).

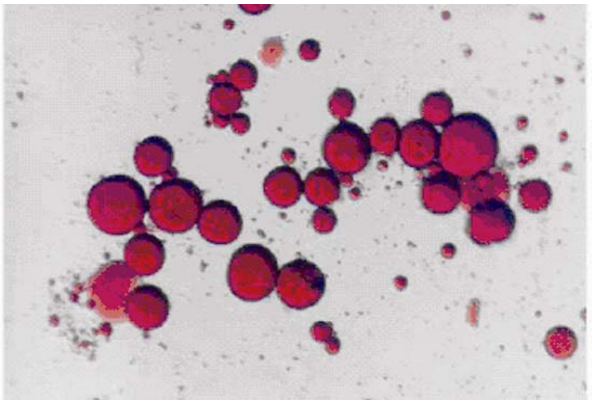

Fig. 2. Adipocytes were stained by oil red O (magnification × 100).

colorimetric assay in 2, 4 and 6 days of culture experiments. Briefly, 50 milligram of MTT [3-(4,5-dimethylthiazolyl) - 2, 5 –diphenyl - tetrazolium bromide, Sigma] was dissolved in 10 ml PBS buffer to prepare MTT stock solution, 500 µl of this solution was added to each well of a 24-well cell culture plate and plates were incubated for 7 h at 37 °C.

Supernatant was removed and 500 µl of 0.04 N HCl in isopropanol was added to each well of a 24-well cell culture plate for 30 minutes. Then samples were transferred from each plate into a 96-well micro plate before reading optical density at 580 nm with an ELISA plate reader (Lab systems Multiscan RC). A standard curve was prepared utilizing a known concentration of cells before each experiment. The MTT test assesses cell metabolism based on the ability of the mitochondrial succinate–tetrazolium reductase system to convert the yellow compound MTT to a blue formazan dye. The amount of dye produced is proportional to the number of live metabolically active cells (19). The percent proliferation (%P) of MCF-7 breast cancer cell in mono and co-culture was calculated according to the following formulas (19).

$$\%P_{monoculture} = [OD_{monoculture} - OD_{day\ 0} / OD_{day\ 0}] \times 100$$

$$\%P_{co\text{-}culture} = [OD_{co\text{-}culture} - OD_{day\ 0} / OD_{day\ 0}] \times 100$$

Histologic and morphologic examination

Collagen gels that contained cultured cells were fixed with 10% formalin solution, embedded in paraffin wax, and routinely processed. The deparaffinized sections were stained with Oil Red O and Mayer's hematoxylin. We performed morphological analyses of cultured cells by light microscopy of stained sections from the cell layer gel obtained from ten blocks in each of the various conditions (18). The images were obtained using a light microscope (Olympus 1 × 70, Japan) and the Magnifier imaging system.

Statistical analysis

Statistical comparisons among several groups were done using one-way analysis of variance, followed by the Tukey test for multiple comparisons among the groups.

RESULTS

Proliferation of culture cells

MCF-7 cell line was grown in mono and co-culture with mitomycin C treated adipocyte and preadipocyte for 6 days and cell proliferation were assessed by MTT absorbance.

Effects of preadipocyte cells on the proliferation of MCF-7 cells

Preadipocytes decreased the proliferation of MCF-7 cells in the co-culture compared to the MCF-7 cells monoculture ($P < 0.05$). On co-culture, the preadipocytes were found to have decreased the proliferation of MCF-7 cells about 10%, 25% and 35%lesser than MCF-7 cell monoculture in 2, 4 and 6 days after cell culture respectively (Fig. 3, 4).

Effects of adipocytes on the proliferation of MCF-7 cells

In contrast to the effects of preadipocytes on MCF-7 cells proliferation, adipocytes increased the proliferation of MCF-7 cells in the co-culture compared to the MCF-7 cells monoculture ($P < 0.05$). On co-culture, the adipocytes were found to have increased proliferation of MCF-7 cells about 10%, 18% and 20% more than MCF-7 monoculture in 2, 4 and 6 days after cell culture respectively (Fig. 3, 5).

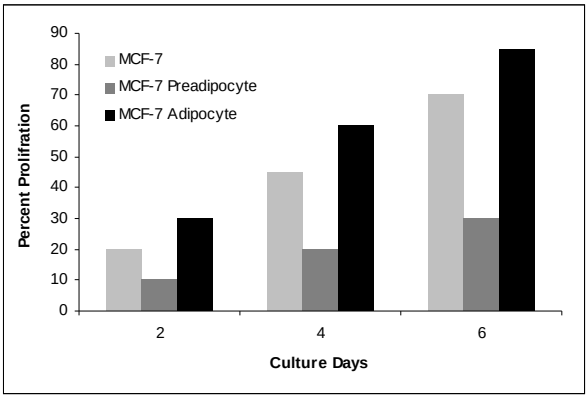

Fig. 3. Growth responses of MCF-7 cells were plated at densities (1×10^4 / well) with or without mitomycin C treated adipocytes or preadipocytes (1×10^5 / well) in collagen gel matrix for 6 days. The MTT assay was performed on 2, 4 and 6 days of culture. Results are expressed as the percentages of cell number. Monocultures of MCF-7 cells were used as a control. Data are expressed as mean ± SEM of triplicate incubation.

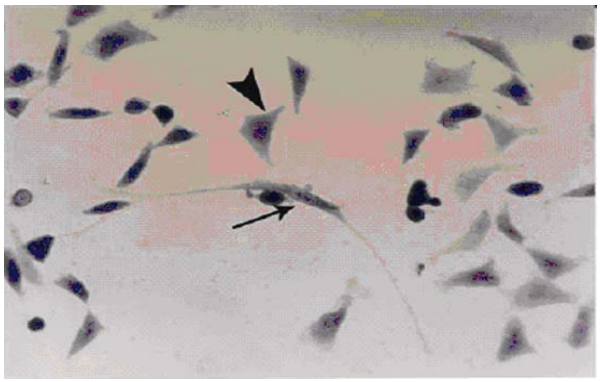

Fig. 4. Co-culture MCF-7 (arrow head) with preadipocyte (arrow) were stained by oil red O and hematoxylin (magnification × 100).

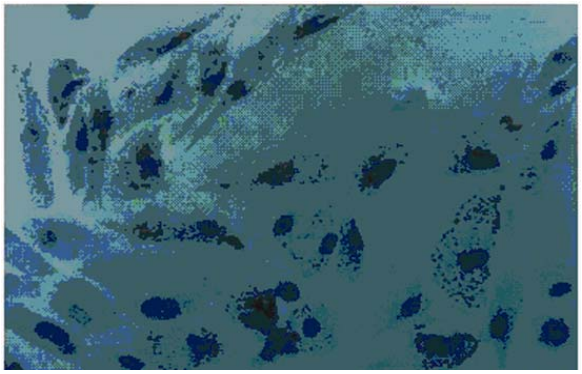

Fig. 6. Monoculture MCF-7 (arrow head) with adipocyte were stained by oil red O and hematoxylin (magnification × 100).

Comparison of MCF-7 cells proliferation in co-culture with adipocytes and preadipocytes

There is a significant difference between proliferation of MCF-7 cells in co-culture with adipocyte and preadipocytes ($P < 0.05$). Proliferation of MCF-7 cells in co- culture with adipocytes were 20%, 43% and 55% more than MCF-7 cells in co-culture with preadipocytes after 2, 4 and 6 days of culture respectively (Fig. 3, 4, 5)

Morphology of MCF-7 cells

MCF-7 breast cancer cell line adheres to mature adipocytes and organized around of spherical adipocytes. MCF-7 cells co-cultured with adipocytes had larger lipid droplets than those of the monoculture. MCF-7 cell line did not show organization of tubular structure with preadipocytes and were separated from it completely (Fig. 3, 4, 5, 6).

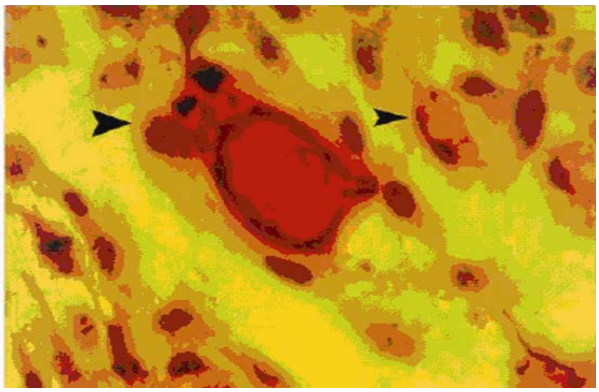

Fig. 5. Co-culture MCF-7 (arrow head) with adipocyte were stained by oil red O and hematoxylin (magnification × 100).

DISCUSSION

The growth of primary and metastatic carcinomas is not simply the result of autonomous growth of malignant epithelial cells, but it requires the interaction between the various cell types within the tumor, including neighboring normal epithelial and stromal cells (20-23). A convenient way to study the complex mechanisms underlying carcinoma-stromal interactions *in vitro* is through the reconstitution of cellular behavior in a 3-D environment obtained by growing cancer cells and stromal cells into solidified collagen gel (18).

Adipose tissue, which exists abundantly in the breast, consists of adipocytes and preadipocytes. These stromal cell types are likely to play vital roles in normal and pathological states of the breast through the paracrine and endocrine effects of secreted adipocyte cytokines. However, few investigations were done about the interaction between primary isolated mature adipocytes and breast cancer cells (18). One of the reasons for this subject is the difficulty in culturing mature adipocytes, which have large lipid droplet and thus do not attach to the surface of the culture dish due to their buoyancy in the medium (24, 25). A number of published studies have provided evidence that adipocytes and preadipocytes surrounding neoplastic cells are undoubtedly involved in tumor growth regulation (9, 26). In this study, we have examined the effect of human adipocytes and preadipocytes on proliferation of MCF-7 breast cancer cell line in a collagen matrix gel. In particular, we have

demonstrated that preadipocytes inhibited the proliferation of the MCF-7 cells in co-culture experiments, while adipocytes increased the proliferation of the MCF-7 cells.

Although few investigations were done about the interaction between adipocytes and preadipocytes with breast cancer cells (18) but there is a controversy in the published literature about the effects of adipocytes and preadipocytes on tumor growth. Johnston et al. demonstrated that murine 3T3-L1 preadipocytes stimulate the growth of normal breast epithelial cells and inhibit the growth of MCF-7 breast cancer cells (27). Chamras *et al.* suggested that murine 3T3-L1 preadipocytes stimulated breast cancer cell growth but in contrast, after differentiation the murine 3T3-L1 preadipocytes to mature adipocytes inhibited clonal growth of breast cancer cell lines (28). Rahimi *et al.* indicated that murine 3T3-L1 preadipocytes spontaneously activate their own secreted TGF-beta, whereas mature adipocytes do not, and suggest that activation of TGF-beta has a potent negative regulatory effect on adipocyte differentiation and tumor growth (29). Manabe *et al.* announced that primary isolated human adipocytes promote the growth of MCF-7 breast cancer cells, whereas primary isolated human preadipocytes inhibit the proliferation of them (18). Lyengar *et al.* demonstrated that murine adipocytes-secreted factors can affect on mammary tumor growth (9).

In this study, we have observed a specific growth promotion of MCF-7 breast cancer cell line when co-cultured with primary isolated human adipocytes but primary isolated human preadipocytes inhibited proliferation of MCF-7 cells. It is conceivable that the different results among these studies may originate from different culture systems and growth assay methods. It also seems likely that primary isolated preadipocytes may show biological phenotypes that differ from those of murine 3T3-L1 preadipocytes.

In agreement with a number of previous works (18, 29, 30), we have observed same morphological changes in the MCF-7 cells. MCF-7 breast cancer cell line organized around of adipocytes but separated from preadipocytes completely. In addition, the MCF-7 cells that co-cultured with adipocytes contain large and rich lipid droplets within their cytoplasm. Our current results support the recent evidence that a large amount of adipose tissue is closely associated with high cellular proliferation of breast cancer in obese women (31). In conclusion, we have shown that subcutaneous adipocytes increase the proliferation of MCF-7 breast cancer cell line but subcutaneous preadipocytes have opposite effects. This suggests that the prevention and therapy of breast cancer may be targeted not only to cancer cells, but also to adipocytes themselves. Future studies will investigate the prevention and therapy of breast cancer via inhibition of preadipocytes differentiation and stimulation of adipocyte dedifferentiation.

Conflict of interests

The authors declare that they have no competing interests.

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