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Full Length Research Paper

A novel and alternative *in vitro* method using microwave to study the epithelial-stromal interactions

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The goal of the present work was to obtain a simple and reproducible experimental model that would maintain the characteristics of the extracellular physiological environment of breast epithelial cells, both in factors as well as stromal structure, on which we could grow and evaluate changes of normal and tumor breast cells. 3T3-L1 pre-adipocytes (breast stromal cell model) were cultured and irradiated in a microwave oven at different times and potencies. In order to lose their proliferation ability, cells had to be irradiated twice at 650 Watts with a two-minute pulse each. The characteristics of the treated stromal support were analyzed for cell morphology, presence of DNA and proteins. We then evaluated on this support the effect on proliferation and migration of both normal and tumor - murine and human - breast epithelial cells. Both cell types increased their proliferation, while only tumor cells increased migration, thus improving their metastatic capacity. We believe this is a new and simple experimental method of studying epithelial-stromal cell interaction.

Key words: Cancer, epithelial-stromal interactions, extracellular matrix, microwave oven, tumor epithelial cells.

INTRODUCTION

A biological system cannot be studied without analyzing the environment, both cellular and extracellular matrix that surrounds it. In this respect, when a cell line is cultured on plastic dishes, a diversity of signals is lost and one can only partially extrapolate the findings to the *in vivo* situation. For this reason, so many different culture conditions have been developed, such as surfaces coated with extracellular matrix (ECM) components or cell feeder layers. One must consider that many cells in tissues are in contact with components of basement membranes, including collagen IV, laminins, heparin sulfate proteoglycans, and growth factors with different biological activities that will condition cell response and, so extracts of basement membrane tissue have been used to study cell-cell or cell-environment interactions.

In cancer, the existence of both mesenchymal-epithelial and epithelial-mesenchymal transitions seems to have a profound influence in the development and progression of the disease (Mani et al., 2008).

The stroma plays a fundamental role in the support and growth regulation of epithelial cells, affecting in great manner the growth and metastatic capability of tumor cells (Rahimi et al., 1994).

Tumor progression occurs within a micro-ecosystem in which carcinogenic and stromal cells exchange different components that promote growth and cell migration. Additionally, a reciprocal influence of both cell types has been postulated to occur through cell to cell contact (Zidar et al., 2002; Julianelli et al., 2007), and by the production of components of the extracellular matrix (Donjacour and Cunha, 1991; Nelson and Bissell, 2006). In addition, the invasion of tumor cells over healthy tissue is promoted through local proteolysis of this matrix, which demonstrates that the ECM besides being a support system plays an essential role in the regulation of cell size, transport and migration (Reichardt, 1993; Nelson and Bissell, 2006).

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Many experimental works use conditioned media obtained from cultured stromal cells in order to evaluate changes in the growth and proliferation of epithelial cells (Kaminski et al., 2006); nevertheless, working with conditioned media has the limitation of providing only soluble factors, while losing the supportive function of the stroma. Additionally, the availability of cell cultures established directly from patient tumors is an important tool in studying cancer biology (Wang et al., 2001). To better understand cancer cell biology and to develop new treatment strategies, there is a need to obtain simple techniques for tumor cell cultures.

The use of feeder cells currently remains optimal for the expansion of epithelial stem cells (Notara et al., 2007). Murine 3T3-L1 feeder cells are commonly used for this purpose. Several authors have shown the usefulness of irradiated mouse fibroblasts as an adequate stromal support for epithelial tumor cell cultures (Wang et al., 2001; Attard et al., 2009). In a previous report (Zizola et al., 2007) our laboratory showed the effect of coating plastic culture dishes with conditioned medium obtained from these 3T3-L1 pre-adipocytes on cell adhesion and proliferation.

The major requirements for cells that are used as a feeder layer are a low rate of cell proliferation and the ability to support the growth of cells to be cloned (Grigoriev et al., 1996). Microwave irradiation has been found to be effective in destructing pathogens in sewage sludge biosolids (Hong et al., 2004). On the other hand, microwaves generated from small “home-type” ovens are effective in sterilizing plastic tissue culture vessels rapidly and inexpensively (Sanborn et al., 1982).

Herein we describe the use of microwave irradiation, a simple technique, to develop natural fibroblast substrate-attached material to study epithelial-stroma interactions and, in the future, to easily develop cancer primary cell cultures from tumor tissue samples.

MATERIALS AND METHODS

Reagents

Reagents were obtained from Sigma Chemical Co (St. Louis, MO, USA). All tissue culture flasks, dishes, and multi-well plates were from Falcon (Orange Scientific, Graignette Business Park, Belgium).

Cell cultures

3T3-L1 murine pre-adipocytes (obtained from American Type Culture Collection, Rockville, MD) were incubated in 5% CO_2 atmosphere at 37ºC and grown in flasks up to 80% confluence. Cells were cultured in T-75 flasks in DMEM-F12 medium with 10% fetal bovine serum (FBS).

Depending on the particular experiment, 3T3-L1 cells were grown on 96-well plates, 24-well-plates, or cultured in Lab-Tek flaskettes (Nunc, Naperville, IL) for histological analysis. For cell proliferation assays, cells were grown up to 50% confluence.

NMuMG (murine normal epithelial breast cells; obtained from American Type Culture Collection, Rockville, MD) and LM3 (murine tumor breast cells; kindly provided by Dr. Elisa Bal de Kier Joffé, Instituto Roffo, Buenos Aires, Argentina) MCF7 (human tumor epithelial breast cells; obtained from American Type Culture Collection, Rockville, MD), T47-D (human tumor epithelial breast cells; obtained from American Type Culture Collection, Rockville, MD) and PC3 (human tumor epithelial prostate cells; obtained from American Type Culture Collection, Rockville, MD) epithelial cells were grown either on irradiated 3T3-L1 cells or on plastic in the presence of DMEM-F12 medium with 10% FBS, and proliferation and migration were evaluated on epithelial cell lines.

Preparation of stromal support

Setup of conditions for optimal microwave irradiation

In order to obtain the optimal irradiation conditions of the stromal support, 3T3-L1 cells were grown up to 80% confluence on 96-well plates and irradiated in a home-type microwave oven with a rotational plate and a maximum power of 900W (Sharp Carrousel II ESP sensor, model R-4H84 operating at 2450 MHz, SHARP Electronics Corp., USA) at different times and power settings. In all assayed conditions a beaker containing 100 ml of water was placed inside the oven to absorb the temperature increase produced by the microwave. The temperature of the water was measured as soon as the beaker was removed from the microwave and reached approximately 80ºC. After each irradiation protocol, DMEM-F12 medium with 10% FBS was added, and 3T3-L1 cells were further incubated in a 5% CO_2 atmosphere at 37ºC for 48 h, after which cell proliferation was measured. In order to determine the number of viable cells, a commercial colorimetric kit was used (Cell titer 96 Aqueous One Solution Cell Proliferation Assay, MTS). Results are expressed as percentage of color intensity measured in the spectrophotometer, where 100% corresponds to the value obtained with control cells, incubated without prior irradiation.

Cell proliferation results were compared to those obtained with 3T3-L1 cells lethally irradiated with 60Gy. In this case, cell proliferation was evaluated 30 min after irradiation.

Partial characterization of stromal support

Optical microscopy observation

3T3-L1 pre-adipocytes were cultured in Lab-Tek chamber slide and incubated in a 5% CO_2 atmosphere at 37ºC up to 80% confluence, with complete DMEM-F12 medium. Cells were then washed with PBS, irradiated in the microwave oven and observed under the optical microscope (IMT-2 Inverted Research Microscope Olympus). Another group of cells was dyed with Hematoxylin/Eosine. Additionally, a wound-healing was performed on 3T3-L1 cells cultured in 24-wells plates up to 80% confluence and dyed with Coomassie blue. Controls received the same described treatment, except for microwave irradiation.

Isolation of substrate-attached material

DNA and Protein determination

The protein profile of the material remaining on the culture plates, was compared with that corresponding to 3T3-L1 cells lethally irradiated with 60 Gy (IBL 437C Gamma Irradiator, CIS Biointernational, Cedex, France, CEBIRSA S.A., Buenos Aires, Argentina) (Rheinwald and Green, 1975). Fibroblasts were grown up to 80% confluence and arrested by microwave or gamma irra-diation. Then, fibroblasts were scraped in PBS and the remaining substrate was washed with PBS and then distilled water. Proteins of

substrate-attached material were obtained as previously described (Culp and Buniel, 1976). Proteins were electrophoresed on 5% PAGE-SDS gels and identified by Coomassie blue staining. DNA extraction of tripsinized 3T3-L1 irradiated cells was carried out as previously described (Herrmann et al., 1994). DNA recovered was electrophoresed in 1% agarose gel containing ethidium bromide.

Epithelial cells cultured on top of the stromal support

Epithelial cell proliferation assay

Proliferation of different epithelial cell lines on 3T3-L1 microwave irradiated cells or on 3T3-L1 lethally irradiated with 60 Gy was compared. Either normal (NMuMG), tumor (LM3) murine, tumor (MCF7 and T47D) human breast epithelial cell lines and tumor (PC3) human prostate epithelial cells were each co-cultured on irradiated 3T3-L1 or gamma irradiated 3T3-L1 stromal supports. 5,000 cells of each cellular type were plated on 96-well plates with complete DMEM-F12 medium with 10% FBS for 24 h to permit the cells adhere to the stromal supports. After this time, the medium was replaced and proliferation was measured after 24 h using MTS assay as previously described. Results are expressed as absorbance at 490 nm.

Epithelial cell migration assay ("wound-healing")

The effect of the stromal support on the motility of NMuMG and LM3 cell lines was evaluated by means of a wound-healing assay. 3T3-L1 cells were plated on 24-well plates, grown up to 80% confluence and then irradiated in the microwave oven. 1.5×10^5 epithelial cells/ ml were plated on top of the stromal support or plastic (control), in 10% FBS medium. When both epithelial cell lines reached 100% confluence, approximately 400 µm wide parallel wounds were performed with a plastic micropipette tip. Photographs were taken 0 and 6 h after performing the wound in order to determine the degree of healing of the original wound (Camera Olympus SC35 Type 12). An image-analysis program (Image J, NIH, Bethesda, MD) was used to quantify the observed differences.

Statistical analysis

Data shown are mean ± SEM. The statistical significance between two mean values obtained for two different experimental conditions was calculated by the Student t-test. The criterion for statistical significance was $p < 0.05$.

RESULTS

Setup of optimal conditions for microwave irradiation

Different exposure times of 3T3-L1 cells to irradiation were assayed at different potencies in order to find the condition under which a low rate of cell proliferation (almost none) could be attained. Figure 1 shows that this condition was reached with 2 min pulses at a potency of 650 W. With this condition, 3T3-L1 cell proliferation was only 7.8 ± 5.3% of the control value attained 48 h post-irradiation.

Comparison of microwave and gamma irradiation on arrest of cell growth

Given that different authors use gamma irradiation to arrest cell growth in order to obtain an adequate stromal support, we wished to compare 3T3-L1 cell proliferation on cultures irradiated with microwaves with those obtained from gamma rays-irradiated cells. Figure 2 shows that 30 min after gamma ray irradiation, 3T3-L1 cells presented a slight but significant increase of proliferation (121 ± 13% compared to control), while microwave-irradiated cells presented an almost complete inhibition of proliferation (17 ± 3% compared to control) at this same time. This value did not change when 500 ml of water instead of 100 ml were added in the beaker that was placed in the oven during irradiation to absorb the increase in temperature.

Cell observation with optical microscopy

Figure 3 shows the differences observed under the optical microscope between non-irradiated and irradiated cells (A vs. B) after staining with hematoxylin-eosin (C vs. D). A decrease in the number of cells attached to the support as well as a loss of typical 3T3-L1 cell histo-architecture was found. A cell layer was removed by means of a pipette tip and a marked staining of the substrate with Coomassie blue after irradiation, was observed even in the area were the wound was performed (Figure 4). Therefore, even though morphological alterations were observed in the irradiated cells, stromal protein products were conserved after irradiation.

Isolation of substrate-attached material

Recovery of DNA and proteins

The next step was to evaluate the presence of DNA and proteins in the stromal substrate. To do this, we grew 3T3-L1 cells on tissue culture dishes, irradiated them in the microwave oven and collected both the DNA and the protein products that were present. DNA was quantified and loaded in an agarose gel. Electrophoresis was performed and the resulting gel was dyed with ethidium bromide (Figure 5A). DNA quantities were 100-fold lower than those corresponding to non-irradiated cells. Additionally, protein products attached to the support were isolated, loaded on 5% polyacrylamide gels, that allow separation of high molecular weight proteins typical of cellular stroma, and electrophoresis was performed. The gel was then dyed with Coomassie blue. Along with the material isolated from the stromal substrate, we loaded on the gels material extracted from non-irradiated (control) and gamma ray-irradiated cells (60 Gy). Even though Coomassie blue staining does not show if proteins have

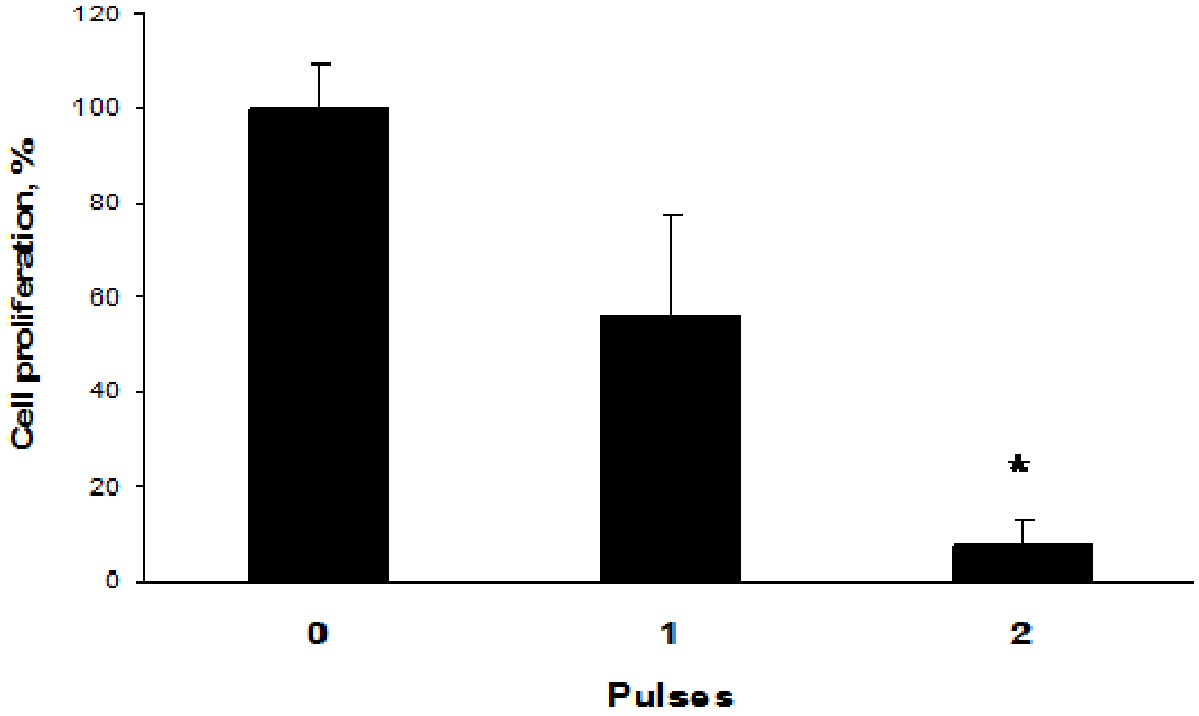

Figure 1. Setup of optimal irradiation conditions to arrest the proliferative capacity of 3T3-L1 cells. 48 h after microwave irradiation, cell proliferation was measured with MTS, using a commercial colorimetric method. The optimal condition to generate a stromal support with low proliferative capacity consisted on 2 min each, pulses at a potency of 650 W, with a 1 min inter-pulse interval. Data are shown as means ± SEM (n = 3, each experiment in 6 replicates). *p < 0.05 compared to the control.

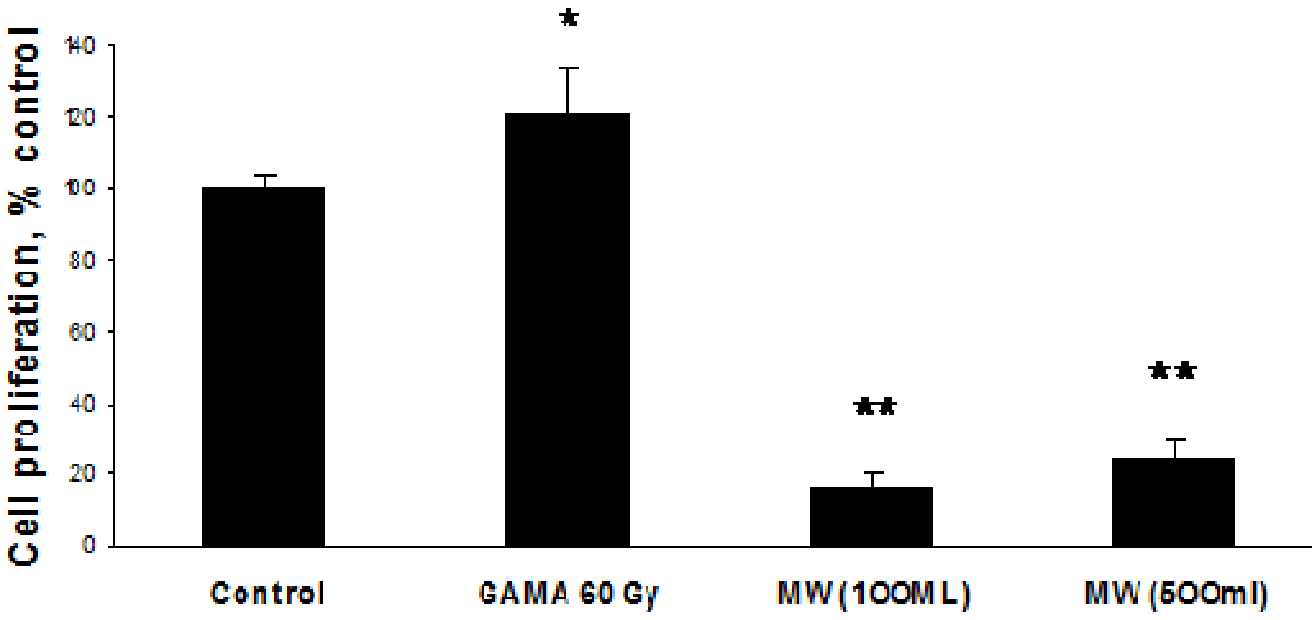

Figure 2. Comparison of gamma and microwave irradiation on cell growth. The proliferation of 3T3-L1 cells was measured 30 min after gamma ray (60 Gy), or microwave irradiation. Cell proliferation increased significantly compared to control after gamma irradiation, while decreasing drastically after microwave irradiation. Different water volumes (MW) in the microwave oven did not modify this result. Data are shown as means ± SEM (n = 3, each experiment performed in 6 replicates). *p < 0.05 and **p < 0.001 compared to the control.

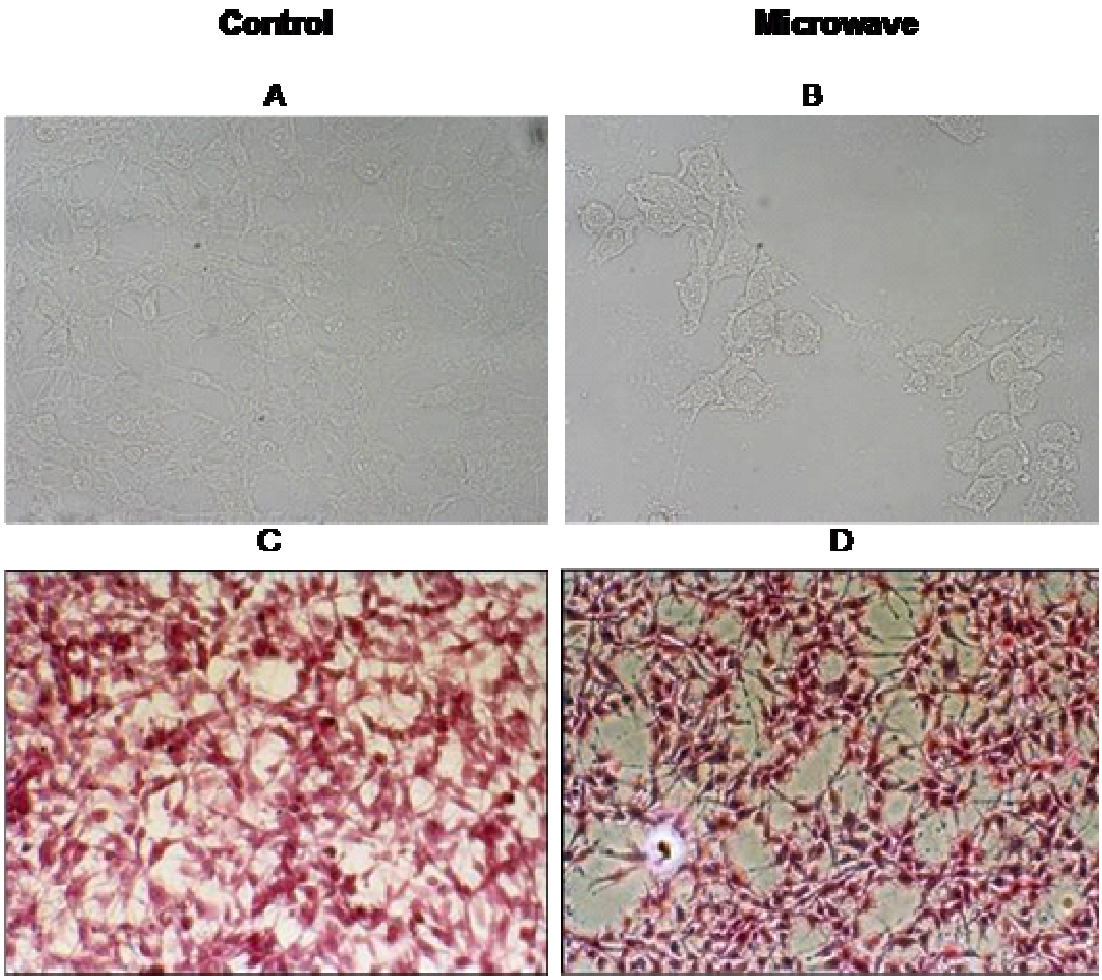

Figure 3, Morphological changes of 3T3-L1 cells after microwave irradiation. Cells were cultured up to 80% confluence, irradiated and then observed under the optical microscope (B, x400). Another group was dyed with Hematoxylin/eosine (D, x200). In both cases, controls consisted of non-irradiated cells (A, x400 and C, x200). Images are representative of 3 different experiments.

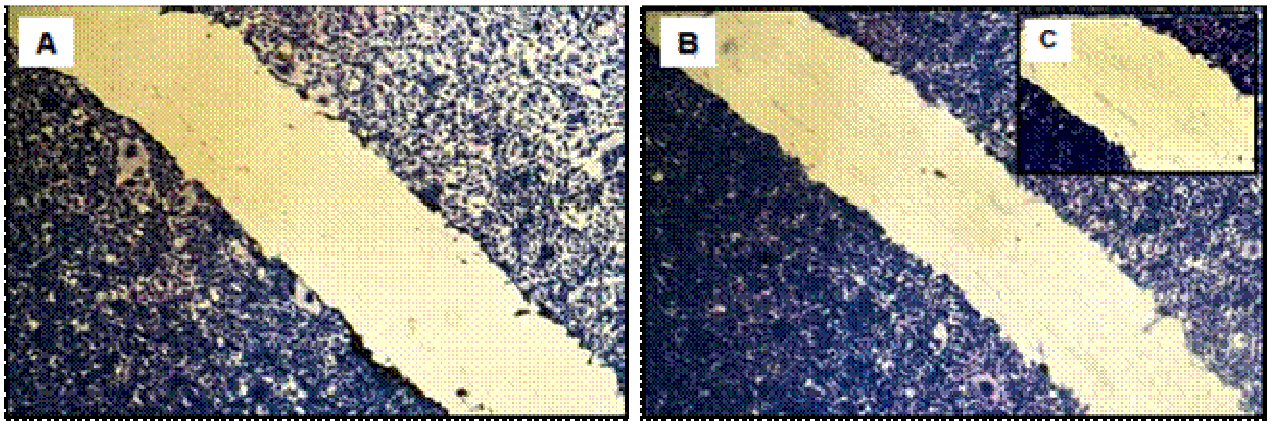

Figure 4. Presence of protein products attached to plastic after irradiation of 3T3-L1 cells. Cells were grown up to 80% confluence and irradiated with microwaves. A wound of approximately 400 µm was then performed and Coomassie blue was added. A marked staining of the substrate is observed for non-irradiated cells (A, x100), as well as after irradiation (B, x100 and C; insert x400). Irradiated cells also showed some amount of staining in the wound area (B and C vs. A).

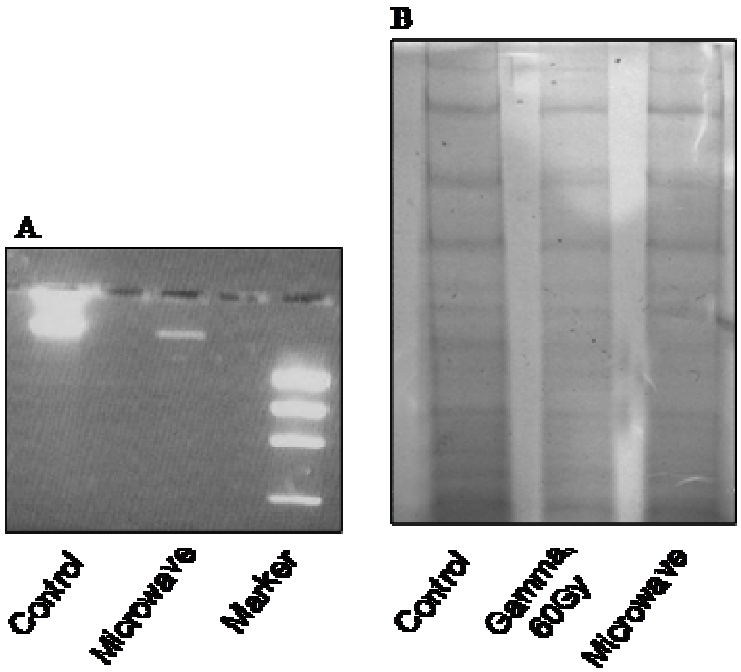

Figure 5. Analysis of DNA and protein products present in the stromal support as a result of 3T3-L1 microwave irradiation. Cell products that were attached to the substrate were collected after 3T3-L1 microwave irradiation and loaded in agarose (A) and 5% polyacrylamide gels (B). A 100-fold decrease of DNA was observed compared to control cells (A). Nevertheless, protein profiles obtained from the SAM were similar to those obtained from control cells and gamma ray irradiated cells.

biological activity, Figure 5B indicates that the resulting protein profiles from 3T3-L1 cells are conserved after microwave irradiation.

Proliferation of normal (NMuMG) and tumor epithelial cells (LM3, MCF-7, T47D and PC3) grown on top of 3T3-L1 microwave-irradiated cells or 3T3-L1 Gamma-irradiated cells

Once the irradiation protocol for the 3T3-L1 cells was established, and the resulting stromal support was partially characterized, we evaluated the effect of the support layers on the growth of NMuMG (murine normal epithelial breast cells), LM3 (murine tumor breast cells), MCF7 (human tumor epithelial breast cells), T47-D (human tumor epithelial breast cells) and PC3 (human tumor epithelial prostate cells). There are not significant differences in absorbance between different tumor epithelial cells lines grown on microwave-irradiated (LM3 0.41 ± 0.02; MCF-7 0.82 ± 0.10; T47D 0.10 ± 0.01; PC3 1.03 ± 0.13) vs gamma-irradiated (LM3 0.44 ± 0.03; MCF-7 0.85 ± 0.04; T47D 0.13 ± 0.02; PC3 0.87 ± 0.16) stromal support. However, we observed increased proliferation of normal epithelial NMuMG cell line growing on 3T3·-L1 gamma irradiated (0.43 ± 0.02) *vs* microwave irradiated (0.33 ± 0.03) support (*$p < 0.05$) (Figure 6)

Proliferation of normal (NMuMG) and tumor (LM3) murine breast epithelial cells grown on top of microwave-irradiated 3T3-L1 cells

Once the irradiation protocol for the 3T3-L1 cells was established, and the resulting stromal support was partially characterized, we evaluated the effect of the support layer on the growth of normal (NMuMG) and tumor (LM3) murine breast epithelial cell lines. Figure 6 shows an increase in the proliferation of both cell types grown on top of the stromal support (121 ± 5% for NMuMG and 125 ± 9% for LM3) with respect to the same cell lines grown on plastic.

Migration of NMuMG and LM3 grown on microwave-irradiated 3T3-L1 cells

Finally, we evaluated the effect on NMuMG and LM3 cells motility, grown on top of the stromal support, after removing a cell layer with a pipette tip. NMuMG cells

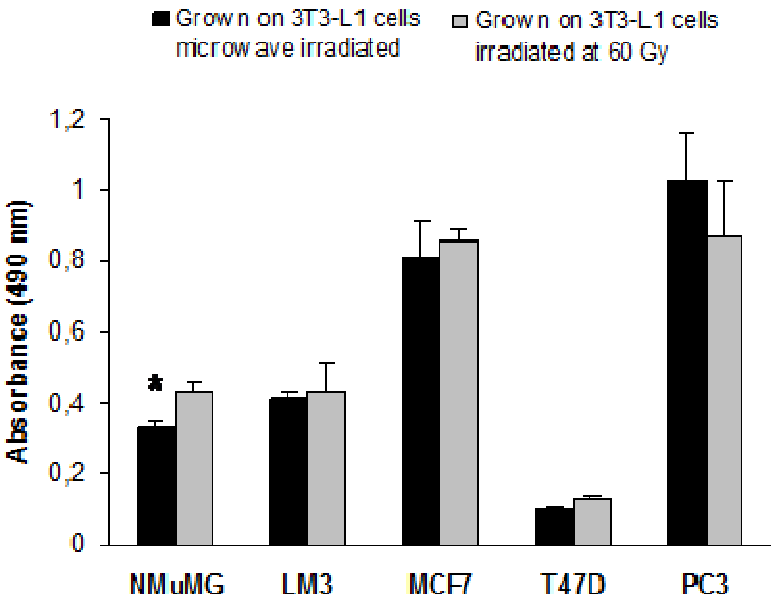

Figure 6. Comparison of proliferation of normal (NMuMG) and tumor (LM3, MCF-7, T47D) breast epithelial cells and tumor (PC3) prostate epithelial cells using gamma irradiated or microwave irradiated 3T3-L1 stromal supports. Cells were grown either on 3T3-L1 microwave-irradiated cells or on 3T3-L1 gamma-irradiated cells for 48 h with complete DMEM-F12 medium. We found increased proliferation of normal epithelial NMuMG cell line growing on 3T3-L1 gamma irradiated *vs* microwave irradiated support. There are not significant differences in proliferation of tumor cell lines tested between both stromal supports. Cell proliferation was quantified using MTS assay. Data are shown as means ± SD (n = 3, each experiment performed in 4 replicates). *$p < 0.05$ compared to cell growth on Gamma irradiated stromal support for the same cell line.

showed no significant differences on migration (wound-healing) after 6 h of performing the wound (Figure 7). Contrarily, LM3 presented a significant closure of the wound (44 ± 2%) with respect to the initial condition (Figure 7). Neither cell line presented changes on the surface of the wound when grown on plastic (Figure 7).

DISCUSSION

Stromal cells are important to stimulate epithelial cell proliferation, and this paracrine effect has been well documented both *in vivo* and *in vitro* assays (Brattain et al., 1982; Epstein and Kaplan, 1979). Different authors have shown that ultraviolet-irradiated fibroblasts increased the plating efficiency of breast epithelial cells and clonal proliferation (Smith et al., 1981), the rate of human tumor growth *in vivo* (Camps et al., 1990), while irradiated 3T3 cell feeder layer may enhance tumor-derived epithelial cell settlement and proliferation from primary breast carcinomas (Wang et al., 2001).

In this work we used 3T3-L1 pre-adipocytes as stromal model, since adipocytes are one of the main stromal cells in human breast tissue. We irradiated these cells with microwaves at different times and potencies in order to find an optimal condition for cell growth arrest, given that this is an essential requisite of any stromal support on top of which other cell types will be grown. We found that two 650 W pulses lasting 2 min each and with a 1 min inter-pulse interval was the optimal condition to achieve our goal. We characterized the stromal support and demonstrated that although cell morphology was altered after irradiation, protein products that were attached to the substrate were maintained. We then tested whether our model was useful for growing normal and tumor - murine and human - epithelial cell lines, and demonstrated that microwave-irradiated 3T3-L1 cells allowed the growth of this cell lines. We found that proliferation rate of several tumor epithelial cell lines was comparable between microwave and gamma (conventional method) irradiated stromal support. Perhaps the different proliferation rates observed in all the cell lines tested might be due to intrinsic characteristics of each cell type, related to specific attachment and interaction with 3T3-L1 fibroblasts. As it was expected, when we compare the two – NMuMG *vs* LM3 - murine cell lines the proliferation level when grown on microwave support was minor in normal than in tumor cell line. Instead, this difference was

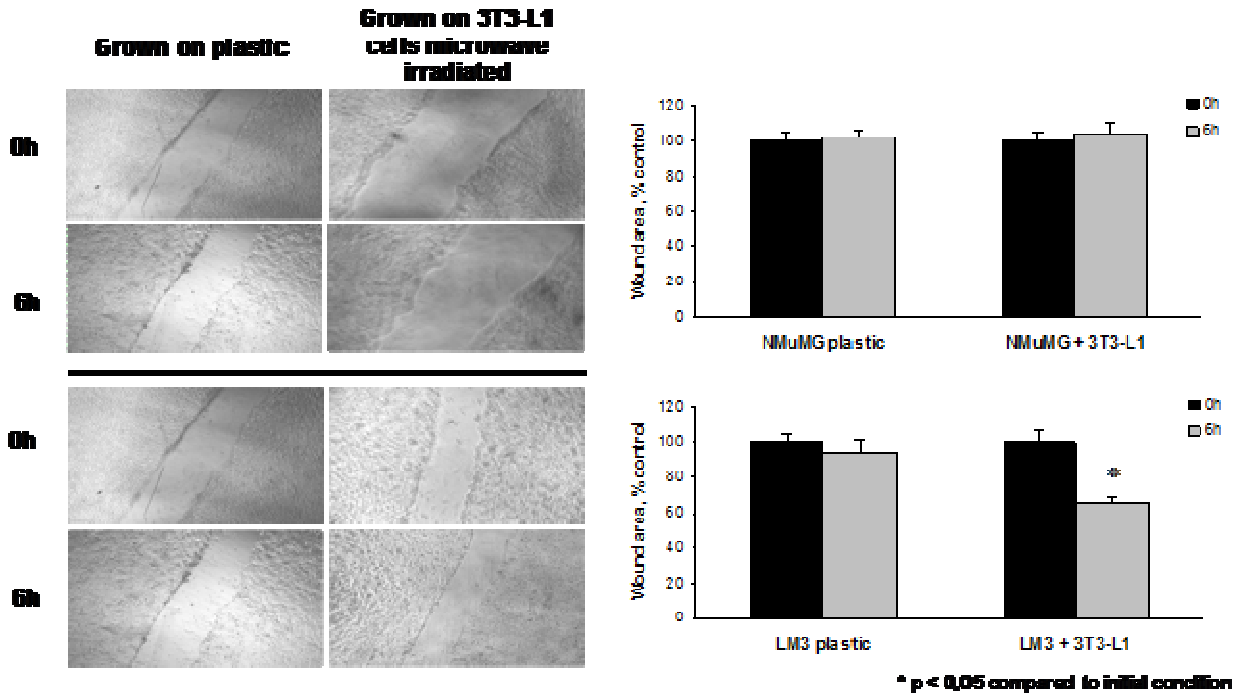

Figure 7. Effect of stromal support on the migration of epithelial cell lines, NMuMG and LM3. Epithelial cells were grown either on 3T3-L1 irradiated cells or on plastic up to confluence with complete DMEM-F12 medium. 400 µm wide wounds were then performed. Photographs were taken 0 and 6 h after performing the wound (Magnification x400). In order to quantify the differences observed in the area of the wounds an image analysis program was used (ImageJ, NIH, Bethesda, MD).

not seen when those cell lines were grown on gamma irradiated support. Furthermore, microwave irradiated support regulated differentially cell migration of normal (NMuMG) and tumor (LM3) murine epithelial cell lines.

The tumor-stroma bidirectional talk consists of multiple dynamic interactions between stromal and neoplastic cells. The complexity of this dialogue cannot be studied directly *in vivo*; while something could be grasped with transgenic models able to focus in a particular factor. *In vitro* models like cell culture are appropriate and used ones in this work.

In conclusion, we present in this work a fast and simple protocol that allows obtaining an adequate stromal support model for the growth of breast and prostate epithelial cells. The use of microwave irradiated fibroblasts for matrix production may be a convenient alternative due to the unavailability of appropriate radiation sources in or near the majority of the laboratories. This model will allow us to extend our analysis of epithelial-stromal interactions to the study of regulation of cell proliferation and migration regulation, and is currently being used with human cell lines as well as human primary cultures from both benign and malignant tissues. Besides, the diversity of cells that can be grown and used as stromal support, allows for a variation in cell culture combinations, otherwise impossible when using commercial products or isolated components of extracellular matrices.

We think that the use of this easy and economical technology could lead to important research assays and help understand tissue, both normal and malignant, development, differentiation and aid disease treatment.

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