Evaluation and Comparison of *In Vitro* Biocompatibility of Poly (Glycolic Acid) and Poly (Lactide-Co-Glycolide Acid) on Mature Spheroids of Tumorigenic and Non-Tumorigenic Cell Lines

Poliglikolik Asit ve Polilaktid Ko-Glikolid Asitlerinin Tümörijenik ve Non-Tümörijenik Matür Sferoidlerde İn Vitro Biyouyumluluğunun Değerlendirilmesi ve Karşılaştırılması

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ABSTRACT Objective: The aim of this study was to evaluate and compare in vitro biocompatibility and poly(glycolic acid) (PGA) and poly(lactide-co-glycolide acid) (PLGA) on tumorigenic and non-tumorigenic mature spheroids. **Material and Methods:** This is an in vitro experimental study. Tumorigenic (C6 glioma, SH-SY5Y, MDAH2774, MCF-7) and non-tumorigenic cells [CRL11372, primary osteoblasts (MCPO)] as well as their mature spheroids were cultured alone as a control group as well as in combination with PGA and PLGA. Total cell numbers, bromodeoxyuridine labeling index (BrDU-LI), apoptosis, morphology, and ultrastructure were evaluated. **Results:** PGA and PLGA significantly decreased the number of SH-SY5Y and C6 glioma cells; MDAH 2774 cells also decreased, but not significantly ($p > 0.05$). Low BrDU-LI ($p < 0.05$) with a high level of apoptosis ($p < 0.05$) at C6 glioma and a high level of BrDU-LI ($p < 0.05$) with a low level of apoptosis at MDAH2774 ($p < 0.01$) were noted. These biopolymers mostly decreased the number of CRL-11372 cells ($p < 0.05$), but indicated an increased apoptosis ($p < 0.01$) and significant BrDU-LI ($p < 0.05$). Biopolymers induced chromatin condensation (typical apoptotic ultrastructure) and vacuolization primarily at SH-SY5Y spheroids but rarely at MDAH-2774 spheroids. This apoptotic ultrastucture was most often observed at MCPO spheroids. PLGA and PGA induced similar BrDU-LI decreases among tumorigenic spheroids ($p < 0.05$), although this decrease was greater at MCF-7 ($p < 0.05$) in the PGA group. PGA primarily decreased BrDU-LI at CRL 11372 ($p < 0.05$), although the decrease was almost identical to that at MCPO for the two biopolymers ($p < 0.05$). A significant attachment affinity was determined at MDAH -2774 and C6 glioma spheroids. **Conclusion:** This study demonstrated the biocompatibility of PGA and PLGA at mature spheroids of tumorigenic and non-tumorigenic cell lines, which changed according to the cell type.

Key Words: Polyglycolic acid; materials testing; spheroids, cellular

ÖZET Amaç: Bu çalışmada, poli(glikolik asit) (PGA) ve poli(laktid-ko-glikolid) (PLGA)'in tümörijenik ve non-tümörijenik matür sferoidlerde biyouyumluluğunun değerlendirilmesi ve karşılaştırılması amaçlandı. **Gereç ve Yöntemler:** Çalışma deneysel olarak planlandı. Tümörojenik (C6 glioma, SH-SY5Y, MDAH 2774, MCF-7) ve non-tümörojenik [CRL 11372, primer osteoblast (MCPO)] hücreler ve olgun sferoidleri tek başına ve biyopolimerler ile birlikte kültüre edildi. Toplam hücre sayısı, bromodeoksiuridin işaretleme indeksi (BrDU-LI), apoptotik indeks, morfoloji ve ultrastrüktür değerlendirildi. **Bulgular:** PGA ve PLGA'nın en fazla SH-SH5Y ve C6 glioma hücre sayılarını azalttığı ve MDAH 2774 hücre sayılarını daha az azalttığı saptandı ($p > 0.05$). Düşük BrDU-LI ($p > 0.05$) yüksek apoptoz ($p < 0.05$) C6 gliomada ve yüksek BrDU-LI ($p > 0.05$) düşük apoptoz ($p < 0.05$) MDAH2774'de ($p < 0.01$) görüldü. Bu polimerlerin hücre sayısını en fazla CRL-11372 ($p > 0.05$) de azalttığı ancak apoptozu ($p < 0.01$) BrDU-LI ($p > 0.05$) artırdığı saptandı. Polimerlerin kromatin birikimini (tipik apoptotik ince yapısı) ve vakuolizasyonu en fazla SH-SY5Y sferoidlerinde ve nadir olarak da MDAH-2774 spheroidlerinde oluşturduğu görüldü. Bu apoptotik inceyapı en fazla MCPO sferoidlerinde saptandı. PLGA ve PGA'nın tümörijenik sferoidler arasında BrDU-LI'de benzer azalmaya neden olduğu ($p > 0.05$), ancak bu azalmanın PGA'daki MCF-7'de daha fazla olduğu görüldü ($p < 0.05$). PGA'nın BrDU-LI'i en fazla CRL 11372'de ($p > 0.05$) azalttığı ancak bu azalmanın MCPO'de her iki polimer için hemen hemen aynı olduğu saptandı ($p > 0.05$). Yüksek tutunma eğilimi MDAH -2774 ve C6 glioma sferoidlerinde görüldü. **Sonuç:** Bu çalışma PGA ve PLGA'nın tümörijenik ve non-tümorijenik matür sferoidlerde biyouyumlu olduğunu ve bu özelliklerinin hücre tipine göre değiştiğini göstermiştir.

Anahtar Kelimeler: Poliglikolik asit; malzemelerin denenmesi; sferoidler, hücresel

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Thermoplastic aliphatic poly(esters) such as poly (glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PLGA) are two well-known biodegradable polymers that play key roles in various pharmaceutical and biomedical research and developmental product programs. These biodegradable polymers hold great promise for overcoming the challenge of limited cell sources for tissue repair as scaffolds and sutures as well as for drug penetration as microcapsules.[1-4]

Previous reports have shown that multicellular tumor spheroids (MTS) represent an intermediary level between monolayer growing cells and tumors in animals and humans. The spheroids more closely mimic the real biologic environment by providing cell-to-cell adhesion and signaling. Consequently, spheroids quite realistically represent the results of the drug effects by including limitations in regard to penetration, distribution, and feedback mechanisms in cell signaling. To date, spheroid cultures have been the most appropriate *in vitro* models to study not only its short-term effects, but more significantly its long-term effects.[5-7]

Spheroids, particularly those derived from liver tissues, have been extensively studied for biocompatibility tests. Tumor cells exhibit extraordinary plasticity and adapt to noxious stimuli, thriving in unfavorable environments. The hallmark of cancer that promotes enhanced adaptation to environmental challenges is a constitutive up-regulation of the cellular response, which lead to an increased resistance to apoptosis.[8-11] Based on studies conducted on rat liver spheroids by Ma et al.[12] and Xu et al.[13] as well as studies on human glioma spheroids by Khaitan et al.,[14] a number of biochemical and functional differences have been observed, changing the time-dependent manners between immature and mature spheroids (e.g., glucose consumption, lactate production, mitochondria number, cell cycle status). Mature spheroids' characteristics change according to cell type based on increased time. Increase of apoptotic and necrotic zones were common. Previous studies further revealed that the stable cell-cell contacts and histological environment in mature spheroids permit and support functional recovery and maintenance *in vitro*.[12-14]

In the field of biomaterial research, a number of studies have examined the three-dimensional co-culture of biopolymers (e.g., PGA, PLA, or PLGA) with hepatocyte, NIH/3T3 fibroblasts, and osteoblast spheroids. However, only a few studies have involved tumorigenic cell lines (i.e., neuroblastoma and glioblastoma cell lines).[15-20] In the few existing studies, single tumorigenic and/or non-tumorigenic cells were co-cultured with these biopolymers over the long term to determine whether they were able to form a spheroid or not.[19,20] These cells in fact formed spheroids with the biopolymers and subsequently continued the co-culture process over the long term. Spheroids became "mature" with these biopolymers.

In the present study, mature spheroids were first formed by culturing them for twelve additional days after their formation, then they were incubated with a quick degradable PGA biopolymer and slow degradable PLGA biopolymer. The aim of this study was to evaluate and compare the *in vitro* biocompatibility of PGA and PLGA in the mature spheroid, and to provide data about the advantages and/or disadvantages of using these biopolymers in the treatment of older patients and older cancer patients in order to fill the gaps in treatment failures.

MATERIAL AND METHODS

This is an *in vitro* experimental study. Tumorigenic [rat glioma (C6 glioma; human breast adenocarcinoma (MCF-7), ovarian carcinoma (MDAH 2774), and neuroblastoma (SH-SY5Y)] and non-tumorigenic [human osteoblast cell line (CRL 11372) and primary mouse calvarial osteoblast] cells, as well as their mature spheroids, were cultured alone and kept in direct contact with the biopolymers. Cell numbers, cell cycle kinetics, apoptosis, morphology, and ultrastructure were evaluated.

MONOLAYER CULTURE

Rat glioblastoma cell line (C6 glioma), human breast adenocarcinoma cell line (MCF-7), human ovarian carcinoma cells (MDAH 2774), SHSY-5Y human neuroblastoma cells and human osteoblast cells (CRL 11372) were supplied by ATCC (Rockville, MD, USA). In addition, with the approval of

the Istanbul University, Institute for Experimental Medical Research (DETAE) Animal Care Investigation Committee, mouse calvaria primary osteoblasts were used for the experiment. All animal experiments were conducted by following the principles of *Guide for the Care and Use of the Laboratory Animals*. C6 glioma and mouse calvaria primary osteoblasts were grown in a monolayer in Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12, Biological Industries, Israel). MDAH 2774 and SHSY-5Y cells were grown in a monolayer in RPMI-1640 medium (Biological Industries, Israel) and in a minimal essential medium (MEM) (Biological Industries, Israel). CRL 11372 cells were grown in DMEM-F12 without phenol red with 2.5 mM L-glutamine and 0.3 mg/ml growth factor (G-418 sulfate, A.G. Scientific Inc., Göttingen, Germany). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Sigma Chemical Co., St Louis, MO, USA). Established cell lines were routinely passaged two times a week and the media were changed with fresh media. Primary osteoblastic cells were controlled daily, and media were changed with fresh media. Semiconfluent cultures were used in all experiments. Cells in semi-confluent flasks were harvested by using 0.05% trypsin (Sigma Chemical Co., St Louis, Missouri), centrifuged after adding a medium for trypsin inactivation and then re-suspended in a medium. Cells with 100% vitality were seeded on microslides in 24-well culture plates containing 1 ml of the medium at a concentration of $1x10^5$ cells/well. Biodegradable polymers fabricated as microflaments from PGA (Biomedical Structures, Slatersville, RI, USA) and PLGA (90:10, Biomedical Structures, Slatersville, RI, USA) were used. Groups were identified as control (n= 3), PGA (n= 3), or PLGA (n= 3). Three different experiments were performed to evaluate various parameters. Cells were incubated with polymers as in the survival studies for 96 hours.[21]

BROMODEOXYURIDINE LABELING INDEX (BrDU-LI) FOR MONOLAYER CULTURE (IMMUNOHISTOCHEMISTRY)

After incubation of cells with biopolymers, 20 µM BrDU (Sigma Chemical Co., St Louis, MO, USA) was added to the medium. Microslides were incubated with this medium for 60 mins at 37°C, rinsed with PBS, incubated with 70% ethanol, rinsed with PBS, and once again incubated with 70% ethanol. Endogenous peroxidase activity was quenched using 0.5% of H_2O_2 in methanol for 15 minutes. Sections were treated with 4 N HCl for 30 minutes at 37°C to denature the DNA. Next, a nonspecific blocking reagent (Ultra-V-Block Lab Vision Co., Westinghouse, CA, USA) was used to prevent nonspecific bindings. Monoclonal mouse anti-BrdU (Lab Vision, Westinghouse, CA, USA) at a dilution of 1:200 was used as the primary antibody for one hour. Secondary antibodies were also used, including biotinylated goat anti-mouse (Lab Vision Co., Westinghouse, CA, USA) for 30 minutes, streptavidin peroxidase for 20 minutes, and peroxidase-compatible chromogen (Lab Vision Co., Westinghouse, CA, USA) for 20 minutes in an incubation box at room temperature. Sections were rinsed with water and counterstained in Mayer's Hematoxylin to enhance nuclear staining for three minutes. BrDU-labeled cells in S phase had a red stained nucleus. For negative controls, the cover slips of the same groups were processed following exactly the same steps except for the primary antibodies. The experiment was performed in triplicate. Two blind expert histologists evaluated at least 3000 cells in multiple high power fields for BrDU labeling index (the number of positively stained cells divided by the counted total number of cells). Photographs were taken using a camera attached to a light microscope.[21,22]

APOPTOSIS

One of the manifestations of apoptosis is the translocation of phosphotidylserine (PS) from the inner membrane to the outer side of the plasma membrane. The externalization of PS was studied using an Annexin V binding assay. Briefly, three different groups (n= 3) were washed with PBS twice and resuspended in a binding buffer containing 0.01 M HEPES, 0.14 mM NaCl, and 2.5 mM $CaCl_2$. A cell suspension (1×10^5 cells in 100 µl) in the binding buffer was incubated with 5 µl of FITC-labeled Annexin V (BD PharMingen, San Diego, CA, USA) and

vital dye propidium iodide for 15 minutes in the dark at room temperature. After incubation, the fluorescence of the cells for propidium iodide (PI) and Annexin V were simultaneously measured in a BD FACSCalibur and analyzed using the instruments' operating software (CellQuest; BD PharMingen). Data acquisition and analysis were conducted using the CellQuest and WinMDI programs.

SPHEROID CULTURE

An *in vitro* multicellular spheroid model was established using the liquid overlay technique. Briefly, once the monolayer cultures became confluent, the cells were trypsinized; and single cells with 100% vitality were cultured over 3% Noble agar-coated (Difco, USA) in six-well culture plates (TPP, Switzerland) containing 5 ml of the specific medium for cells at a concentration of $1x10^6$ cells/well. Spheroid formation manner and time varied according to cell type. Cells formed 40 to 60 spheroids, with diameters ranging from 120 to 300 μm in each well. Spheroids that reached diameters ranging from 120 μm to 300 μm were co-cultured with PGA (Biomedical Structures, Slatersville, RI, USA) and PLGA (10% PLA and 90% PGA; Biomedical Structures, Slatersville, RI, USA) biodegradable polyesters, over 3% Noble agar-coated in six-well culture plates containing 5 ml of the medium. Spheroids were routinely controlled daily and rinsed with fresh medium to prevent aggregation; their media were changed for fresh media as well. Spheroids from the three groups (n= 3) were examined daily and photographed to determine whether they attached to polyesters. Three different experiments were conducted to evaluate different parameters. The alterations of spheroid morphology and ultrastructure were observed under the light microscope (LM) and the transmission electron microscope (TEM).[21]

BROMODEOXYURIDINE LABELING INDEX (BrDU-LI) FOR SPHEROID CULTURE (IMMUNOHISTOCHEMISTRY)

After incubation of spheroids with biopolymers, 20 μM BrDU (Sigma Chemical Co., St Louis, MO, USA) was added to the medium. Spheroids were gently removed from the surface of the solidified agar, and the sections were treated with 0.1% trypsin for antigen retrieval and incubated in 0.3% H_2O_2. The sections were then treated with 4 N HCl and incubated in 0.1 M Borax. Following the washings, a nonspecific blocking reagent (Ultra-V-Block Lab Vision Co., Westinghouse, CA, USA) was used to prevent nonspecific binding. A monoclonal mouse anti-BrdU antibody (1:50; Lab Vision Co, Westinghouse, CA, USA) served as the primary antibody while biotinylated goat anti-mouse antibody (Lab Vision Co., Westinghouse, CA, USA) as a secondary antibody. After washing, peroxidase-conjugated streptavidin (Lab Vision Co., Westinghouse, CA, USA) was applied and aminoethyl carbazole was used as a chromogen. The sections were counterstained in Mayer's Hematoxylin. BrDU-labeled cells in S phase had red stained nuclei. The experiment was performed in triplicate. Two blind expert histologists evaluated approximately 25 spheroids on each slide that had been identified for the calculation of the BrDU labeling index (i.e., the number of BrDU positively stained cells divided by the total counted cells). Mean cell counts immunostained with BrDU were calculated. For negative controls, adjacent sections were processed following exactly the same steps, excluding the primary antibodies.[21,22]

TRANSMISSION ELECTRON MICROSCOPY

Harvested spheroids from three groups (n= 3) were fixed with 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH= 7.2-7.4) and post-fixed in 1% osmium tetraoxide in a 0.1 M sodium cacodylate buffer for 1 hour at 4°C. Spheroids were subsequently incubated in 1% uranyl acetate for 1 hour at 4°C, dehydrated in a graded alcohol series, incubated in propylene oxide for 30 minutes, embedded in Epon 812 (Sigma-Aldrich, Steinheim, Germany) and finally polymerized at 70°C for 20 hours. Samples were cut on a microtome (Leica MR 2145, Heerbrugg, Switzerland); 70 nm thick sections were mounted on copper grids, stained with 5% uranyl acetate, and counterstained with Reynolds's lead citrate. Sections were examined using a Jeol-Jem 1011 transmission electron microscope.[21,22]

STATISTICAL ANALYSIS

Statistical analysis was performed using Sigma Stat program 12.0 (Version 12.0; SPSS Inc., Chicago, IL, USA). The significance of difference was analyzed using the student *t*-test. All data are presented as mean±standard error mean (SEM). Results are the mean of the three different experiments. A p value of less than 0.05 was considered significant.

RESULTS

CELL PROLIFERATION

The effects of the PGA and PLGA biopolymers on a monolayer culture of tumorigenic and non-tumorigenic cell lines from human, mouse, and rat samples for 96 hours were evaluated (Figure 1). The control group of all cell lines exerted healthy growth for the 96 hours of incubation time. In comparison to the control group, both PGA and its copolymer PLGA acted similarly, slightly reducing the number of cells of rat glioblastoma C6 glioma and human breast adenocarcinoma MCF-7 for 72 hours as well as those of human ovarian carcinoma MDAH 2774 and human neuroblastoma SH-SY5Y for 96 hours. At 96th hour, the PGA effects on the number of C6 glioma cells were not different from the control group, in which the percentage of increase was 104% ($p > 0.05$), although PLGA slightly increased the number of cells by 12% ($p > 0.05$). At 96th hour, PGA (107%; $p > 0.05$) and PLGA (114 %; $p > 0.05$) slightly increased the ratio of MCF-7 cells. In comparison to the control group, both PGA and PLGA acted similarly to reduce the number of human osteoblast (CRL 11372) cells for all time intervals and for mouse calvaria primary osteoblasts for 48 hours. No significant difference emerged between the number of cells in the control group and the PLGA-incubated group for the mouse calvaria primary osteoblasts (3%; $p > 0.05$) on the 96th hour (Figure 1). PGA and PLGA induced a significant decrease in the number of SH-SH5Y cells; this decrease was smaller than other tumorigenic groups at MDAH-2774. Two biopolymers led to a significant decrease in the number of CRL11372 cells. PGA and PLGA were more biocompatible with MDAH-2774 cells among the tumorigenic cell lines and mouse calvaria primary osteoblasts among the non-tumorigenic cell lines.

BROMODEOXYURIDINE LABELING INDEX (BrDU-LI) FOR MONOLAYER CULTURE

This section discusses BrDU results of the monolayer culture of tumorigenic and non-tumorigenic cell lines from human, rat, and mouse with or without PGA and PLGA at 24 and 96 hours (Figures 2 and 3). Cells containing nuclei with at least five red grains were considered as BrDU-LI-labeled cells (Figure 2). As shown in the control groups of all cell lines, many of the cells' nuclei were positive for BrDU; this number reduced over time due to the enhanced population of cells consuming the same nutritional pool with BrDU-LI values. Slight reductions of BrDU-LI were observed for both PGA and PLGA, although PGA reduced more than PLGA. Significant versus slight decreases at BrDU-LI were determined at SH-SY5Y and MDAH-2774 cell lines among tumorigenic cells. Meanwhile, a significant decrease was found in the primary mouse calvaria osteoblasts (MCPO) cell line among non-tumorigenic cells. In comparison to the control group, these reductions were not statistically significant for all time intervals ($p > 0.05$) (Figure

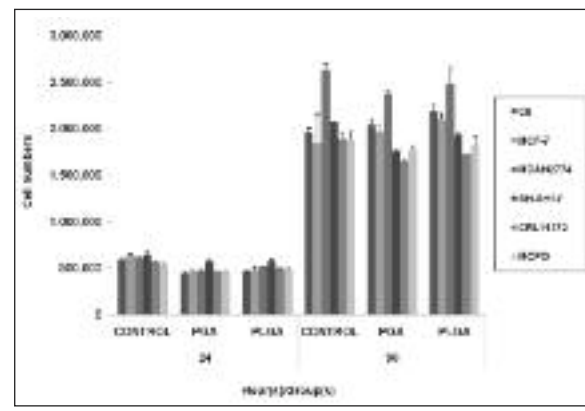

FIGURE 1: Alterations of monolayer cultures of tumorigenic and non-tumorigenic cell lines incubated with PGA and PLGA for 96 hours. Cells were initially plated at a seeding density of 5x105 cells per well. Significant as well as subtle decreases in the number of cells occurred at SH-SH5Y and MDAH-2774 cell lines among tumorigenic cells. On the other hand, significant decreases in number of cells occurred at the CRL11372 cell line among non-tumorigenic cells. Results are represented as mean ± standard error mean (SEM) using the mean of the three experiments.

MCPO: Mouse calvaria primary osteoblasts.

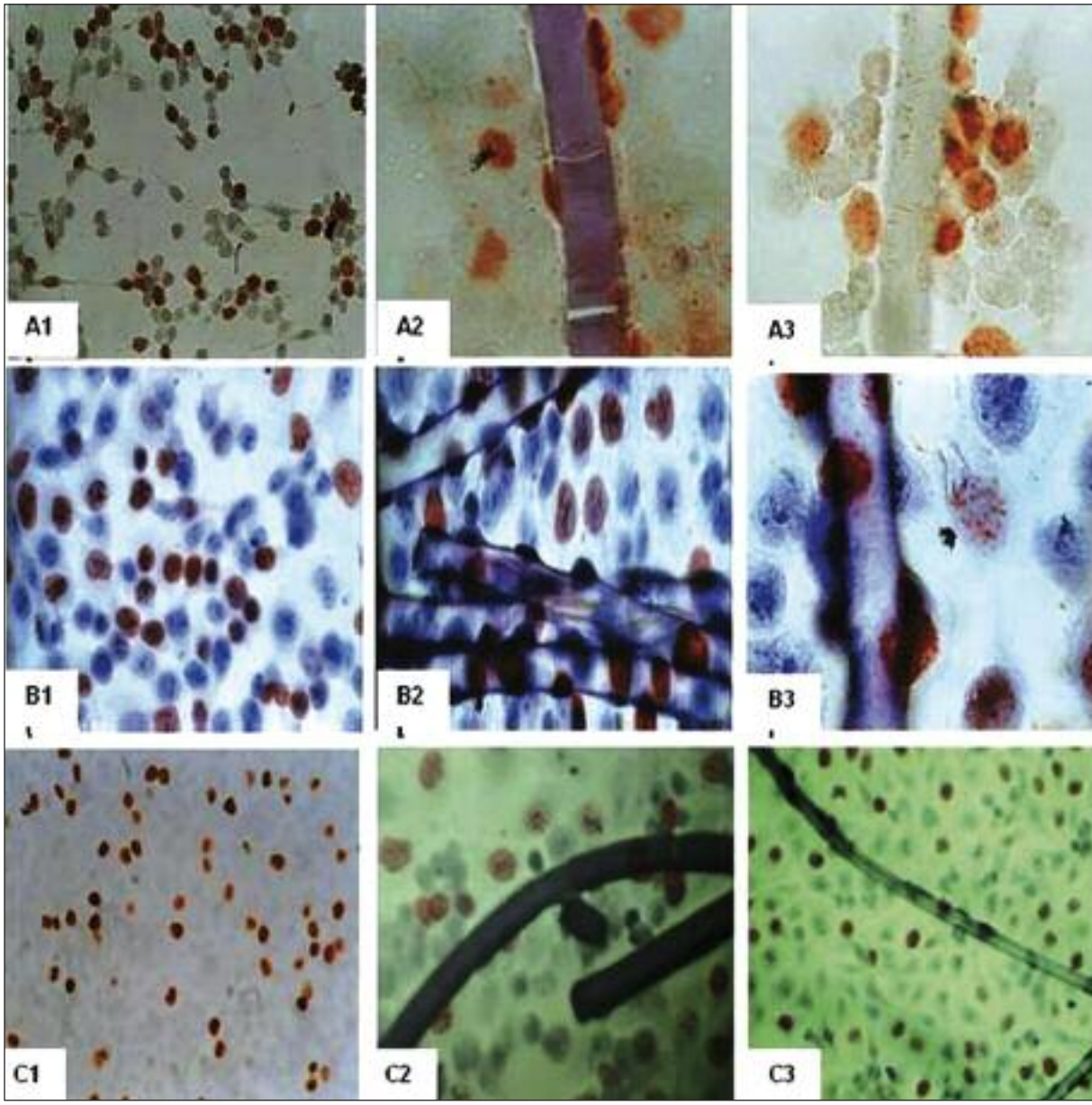

FIGURE 2: Bromodeoxyuridine labeling index (BrDU-LI) micrographs of monolayer cultured C6 glioma, MCF-7, and MDAH2774 cell lines. Positive BrDU-LI represents cells in the S-phase of the cell cycle, determined by the red staining due to the use of AEC chromogen. Cells at the micrograph attached to biopolymers entered the S-phase of the cell cycle; consequently, biopolymers did not negatively affect BrDU-LI. Control, PGA, and PLGA groups are represented by numbers 1, 2, and 3, respectively. **A.** C6 glioma (Original magnification for Control x 40, PGA and PLGA x 100) **B.** MCF-7 (Original magnification for Control x 40, PGA x 100 and PLGA x 40) **C.** MDAH 2774 (Original magnification for Control x 40, PGA x 40 and PLGA x 40).

3). Thus, PGA and PLGA seemed to be more biocompatible with MDAH-2774 cells among tumorigenic cell lines, and in contrast to cell number results, the same was true in mouse calvaria primary osteoblasts among non-tumorigenic cell lines.

BROMODEOXYURIDINE LABELING INDEX (BrDU-LI) FOR SPHEROID CULTURE

Twenty-five spheroids from all experimental groups (including the control group) were evaluated for 96 hours. Cells containing nuclei with at least five red grains were considered as BrDU-LI-labe-

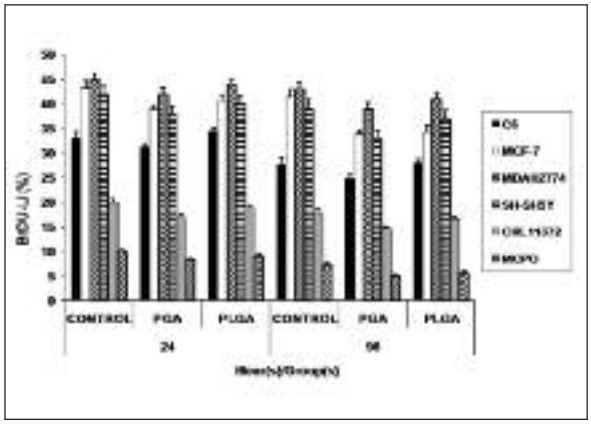

FIGURE 3: Bromodeoxyuridine labeling index (BrDU-LI) results of the monolayer cultured tumorigenic and non-tumorigenic cell lines incubated with PGA and PLGA for 96 hours. Cells were initially plated at a seeding density of 1x105 cells per well. BrDU-LI was calculated by evaluating at least 3000 cell sections in multiple well-labeled high power fields; the result was formulated as the number of positively stained cells divided by the total number of cells counted. Significant and subtle decreases in BrDU-LI were determined at SH-SY5Y and MDAH-2774 cell lines among tumorigenic cells. On the other hand, a significant decrease occurred at the mouse calvaria primary osteoblasts (MCPO) cell line among non-tumorigenic cells. Results are represented as mean ± SEM. Results use the mean of the three experiments.
MCPO: Mouse calvaria primary osteoblasts.

led cells (Figure 3). The BrDU-LI values of C6 glioma, MCF-7, MDAH 2774, and SH-SY5Y were 24%, 19.2%, 26.5%, and 24% respectively for control, 20.02%, 16.14%, 23.06%, and 20.3% respectively for PGA, and 22.1%, 17.3%, 25.38%, and 21.68% respectively for PLGA. Both PGA and PLGA reduced the S-phase fractions when compared to the control group; however, these reductions were not statistically significant ($p > 0.05$). The reduction rates were almost the same among the tumorigenic groups for PLGA; only the reduction rates in MCF-7 were lower for PGA ($p < 0.05$).

The PGA reduction rates were greater than those of PLGA. Like tumorigenic cell lines, PGA and PLGA reduced BrDU-LI values from 14.1% to 9.52% and 11.54%, respectively for CRL 11372 and from 11.2% to 8.02% and 8.55% for mouse calvaria primary osteoblasts. PGA reduction rates were greater than those of PLGA at the CRL 11372 cell line, but these reduction rates were almost the same at the primary mouse calvaria osteoblasts (MCPO). These reductions were not statistically significant ($p > 0.05$) (Figure 4). Thus, PGA and PLGA seemed to be biocompatible with tumorigenic spheroids, whereas PLGA seemed more biocompatible than PGA with non-tumorigenic spheroids.

CELL MORPHOLOGY WITH LIGHT MICROSCOPY

A light microscopic (LM) examination of the monolayer culture revealed that many cells from both tumorigenic and non-tumorigenic groups incubated with PGA and PLGA polymer fibers exhibited characteristics of their specific morphology, just like the control group (Figures 2 and 5). C6 glioma had a typical spindle shape with a centrally located nucleus. Many cells were positively stained for the mouse monoclonal BrDU antibody, attached to polymer fibers and proliferated (Figure 2A); meanwhile, some attached to polymers weakly or did not attach, but continued to proliferate. Likewise, C6 glioma cells MCF-7 (Figure 2B), MDAH 2774 (Figure 2C), SH-SY5Y, human osteoblast cells (data not shown), and mouse calvaria primary osteoblasts exhibited normal morphology and cell-polymer attachment during active DNA synthesis.

SPHEROID MORPHOLOGY WITH LIGHT MICROSCOPY

The manner and time of spheroid formation varied according to cell type; cell-biopolymer attachment time and rate also varied from cell to cell. The attachment affinity of tumorigenic and non-tumorigenic cell-derived spheroids to PLGA seemed to be

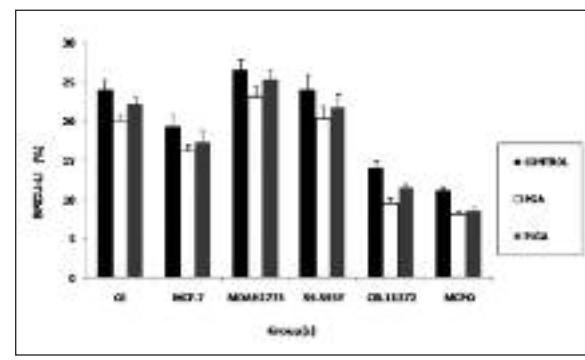

FIGURE 4: Bromodeoxyuridine labeling index (BrDU-LI) results of the spheroid culture of tumorigenic and non-tumorigenic cell lines with or without PGA and PLGA at 96th hour. Cells were initially plated at a seeding density of 1 x 106 cells per well. Approximately 25 spheroids on each slide were used for the calculation of the BrDU-LI, which was formulated as the number of positively stained cells divided by the total number cells. Mean counts of cells immunostained by BrDU were also calculated. Results are represented as mean ± SEM using the mean of the three experiments.
MCPO: Mouse calvaria primary osteoblasts.

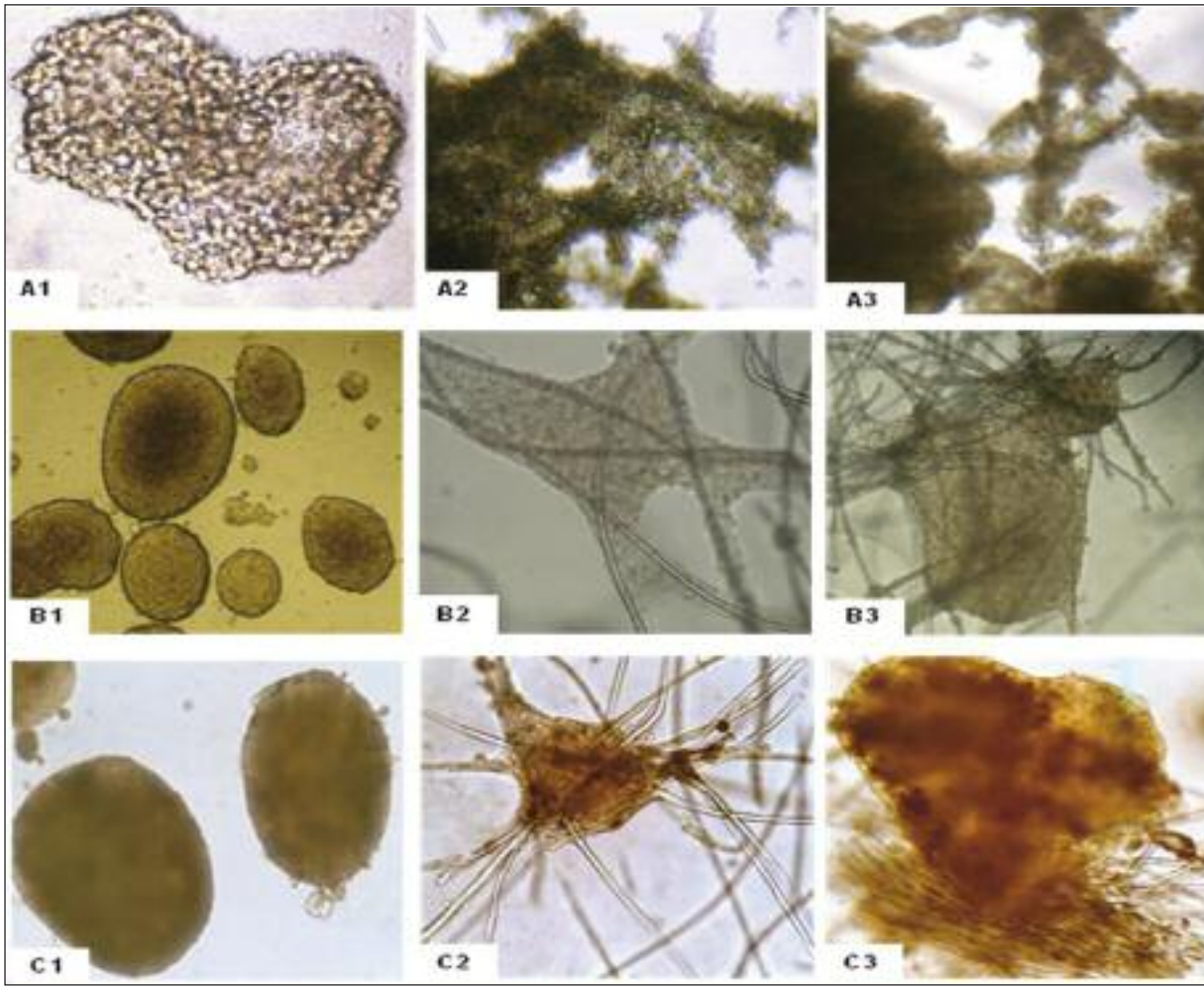

FIGURE 5: Micrographs of tumorigenic (C6 glioma, SH-SY5Y) and non-tumorigenic (primary mouse calvaria osteoblasts) spheroids incubated with PGA and PLGA. Spheroids attached to fibers, lost their characteristic shapes while surrounding the fibers, and filled the gaps between polymers like a tissue constitution. Control, PGA, and PLGA groups of all cell lines are represented by numbers 1, 2, and 3, respectively. **A.** C6 glioma at day 7 (Original magnification for Control x 100, PGA x 40 and PLGA x 100) **B.** SH-SY5Y at day 1 (Original magnification for Control, PGA and PLGA x 40) **C.** Mouse calvaria primary osteoblasts (MCPO) at day 3 (Original magnification for Control x 100, PGA and PLGA x 40). Cells were initially plated at a seeding density of 1x106 cells per well.

greater than to PGA. The attachment of MDAH 2774 spheroids to PGA polymer fibers seemed weaker than C6 glioma spheroids (Figure 5A). Over time, these spheroids attached to fibers more strongly than before and lost their characteristic shapes within the surrounding fibers, filling the gaps between polymers like a tissue constitution (Figures 5B and 5C). The attachment of SH-SY5Y, MCF-7, and osteoblast spheroids to PGA increased during prolonged incubation. Thus, PLGA seemed to be more biocompatible to all types of spheroids than PGA.

APOPTOSIS

Different levels of apoptosis induced by the two biopolymers were evaluated (Figure 6). The order of cell lines, from high apoptotic index to low, was mouse calvaria primary osteoblasts, CRL-11372, SH-SY5Y, C6 glioma, MCF-7, and MDAH2774 for PGA at 24^{th} and 96^{th} hours. In comparison to the control group, the increases in apoptosis were statistically significant for groups of SH-SY5Y ($p< 0.01$), CRL11372 ($p< 0.01$), and mouse calvaria primary osteoblasts ($p< 0.05$) for PGA at 24 and 96 hours. For PLGA, the order was mouse calvaria

primary osteoblasts,CRL-11372, SHSH-5Y, C6 glioma, MCF-7, and MDAH2774 at 24th hour and mouse calvaria primary osteoblasts,CRL-11372, C6 glioma, SH-SY5Y, MCF-7, and MDAH2774 at 96th hour. In comparison to the control group, the increases in apoptosis were statistically significant in MCF-7, SH-SY5Y, and CRL11372 groups ($p< 0.05$) for PLGA at 24 and 96 hours. The greatest increase in apoptosis for PGA and PLGA was SHSY-5Y and CRL11372 while the lowest apoptotic rate was at MDAH-2774. PGA and PLGA were more biocompatible with MDAH-2774 cells among tumorigenic cell lines and mouse calvaria primary osteoblasts among non-tumorigenic cell lines.

SPHEROID ULTRASTRUCTURE WITH TRANSMISSION ELECTRON MICROSCOPY (TEM)

We discriminated among ultrastructural differences between the control group and incubated biopolymer fibers groups using transmission electron microscopy (TEM) (Figure 7). Mature spheroids were evaluated to determine their dominant cell shape and growth pattern.

TUMORIGENIC SPHEROIDS

SH-SY5Y Neuroblastoma

The control group showed normal morphology, as evident in the fine-textured nuclear chromatin, defined oval heterochromatic nuclei, intact nuclear membrane, proportional organelles distributed in the cytoplasm, tubular structured mitochondria, intact cytoplasmic membrane, and many microvilli being in contact with other cells. PGA and PLGA incubated spheroids showed chromatin condensation typical of apoptotic cells and vacuolization. However, spheroids incubated with PGA showed this appearance more often than those that were PLGA incubated. Some spheroids in both groups were observed to attach to the polymers weakly and were unable to take the shape of biopolymers' surface to constitute a layer-like tissue.

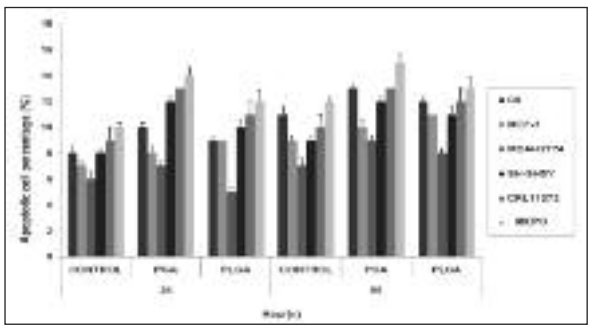

FIGURE 6: The effects of polymers on apoptotic cell percentage of monolayer cultured tumorigenic and non-tumorigenic cell lines. This was discriminated by flow cytometric data obtained using Annexin V-FITC and PI double staining. The percentage of apoptotic cells is plotted (%). Significant and insignificant apoptotic indices were determined at SHSY-5Y and MDAH-2774 cell lines among tumorigenic cells, respectively. On the other hand, a significant apoptotic index was defined at the CRL11372 cell line among non-tumorigenic cells. Results are represented as mean ± SEM using the mean of the three experiments.

MCPO: Mouse calvaria primary osteoblasts.

C6 Glioma

The control group of C6 glioma exhibited normal morphology, characterized by fine-textured ovoid nuclear chromatin, heterochromatic nuclei, intact nucleus membrane, a cytoplasm rich in secretory materials, intact cytoplasmic membrane and microvillus. Mitotic activity was also observed (Figure 7A). It was commonly noted that no marked morphological difference occurred between control spheroids and spheroids incubated with PGA and PLGA. The increase of vacuoles was noteworthy for biopolymer-incubated groups, in which the apoptotic appearance was observed. The number of vacuoles as well as the apoptotic appearance were observed more often in PLGA-incubated spheroids. In addition, some spheroids in both groups attached to the polymers weakly and were unable to take the shape of biopolymers' surface to constitute a layer like tissue (Figures 7B and 7C).

MCF-7

Normal morphology was evident in the MCF-7 control group, as characterized by a fine texture of nuclear chromatin, intact nucleus membrane, a cytoplasm rich in secretory materials, proportional organelles distributed in the cytoplasm, intact cytoplasmic membrane, and microvillus. It was commonly noted that no marked morphological difference occurred between control spheroids and spheroids incubated with PGA and PLGA. The increase of vacuoles was noteworthy for biopolymer-incubated groups while the apoptotic appearance was rarely observed. The number of va-

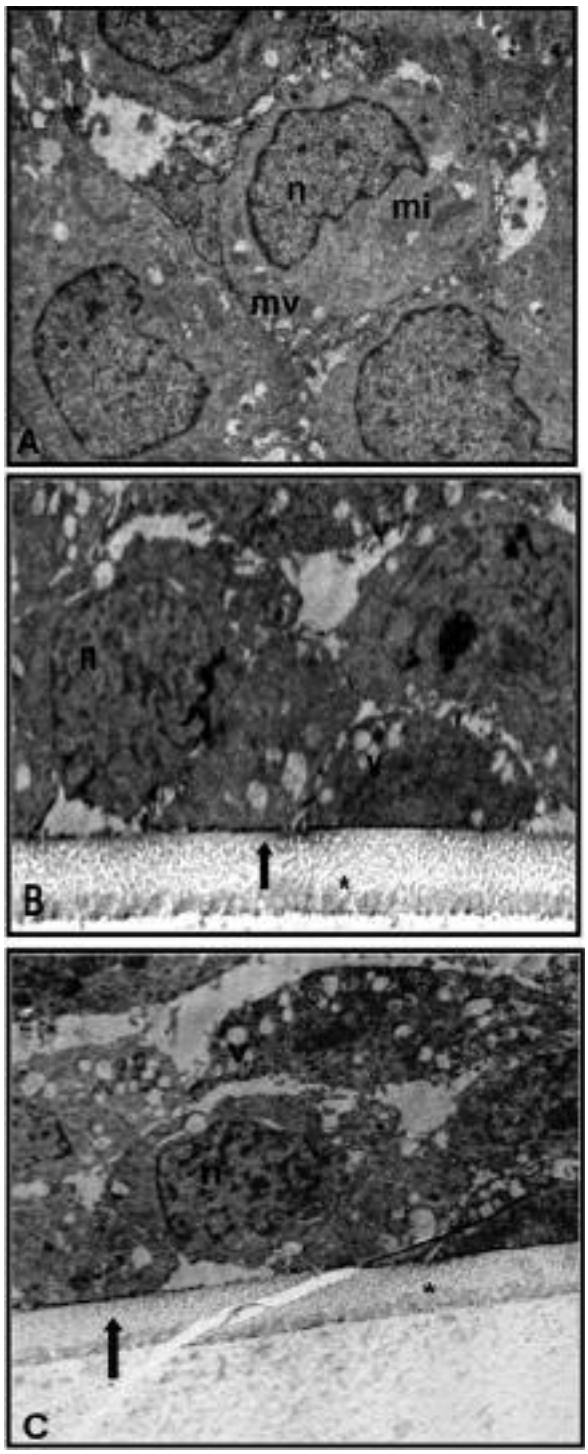

FIGURE 7: Transmission electron micrographs of C6 glioma spheroids. **A.** Control group (Original magnification x 5000) **B.** PLGA group (Original magnification x 6000) **C.** PGA group (Original magnification x 5000) **n:** nucleus, **v:** vacuole, **mi:** mitochondria, **mv:** microvillus, (*): biopolymer surface, one arrow (↑): attachment of spheroids to biopolymers. Unlike PGA groups, vacuolization in PLGA groups was rarely observed. Spheroids of all groups attached to biopolymers; at the attachment points, they lost their oval shape, took the shape of biopolymers' surface, became flat, and constituted a layer-like tissue.

cuoles and apoptotic appearance were almost the same for both PGA- and PLGA-incubated spheroids. Spheroids in both groups attached to the polymers, took the shape of biopolymers' surface, and constituted a layer like tissue.

MDAH-2774

Normal morphology characterized by the fine texture of nuclear chromatin, intact nucleus membrane, a cytoplasm rich in secretory materials, proportional organelles distributed in the cytoplasm, tubular structured mitochondria, intact cytoplasmic membrane, and microvillus was observed in the MDAH-2774 control group. It was commonly noted that marked morphological difference occurred between control spheroids and spheroids incubated with PGA and PLGA. The increase of vacuoles was noteworthy for biopolymer-incubated groups, while the apoptotic appearance was only very rarely observed. The number of vacuoles and apoptotic appearance were almost the same for both PGA- and PLGA-incubated spheroids. Spheroids in both groups attached to the polymers, took shape from the biopolymers' surface, and constituted a layer-like tissue.

NON-TUMORIGENIC SPHEROIDS

Ultrastructural studies of mouse calvaria primary osteoblasts and CRL-11372 indicated that control groups had a normal ultrastructural appearance, namely, a fine texture of ovoid nuclear chromatin, heterochromatic nuclei, intact nucleus membrane, an intact cytoplasmic membrane, and microvillus (Figure 8A). In particular, PGA- and PLGA-incubated spheroids from two cell lines showed chromatin condensation typical of apoptotic cells and vacuolization (Figures 8B and 8C). However, PGA-incubated osteoblasts' spheroids often exhibited this morphology (Figure 8C). Among these two types of osteoblasts, spheroids of mouse calvaria primary osteoblasts showed a marked apoptotic appearance and vacuolization in both PGA and PLGA groups. Some spheroids-especially in the PGA groups-commonly attached to the polymers weakly and were unable to take the shape of biopolymers' surface to constitute a layer-like tissue.

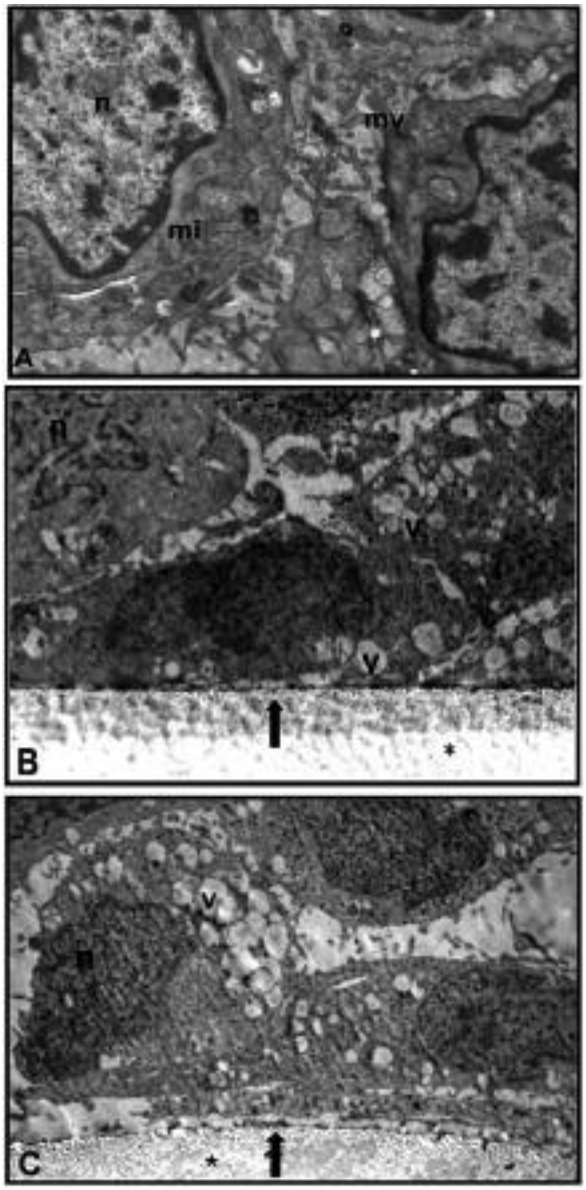

FIGURE 8: Transmission electron micrographs of human osteoblast CRL11372 spheroids. **A.** Control group (Original magnification x 12k) **B.** PLGA group (Original magnification x5000) **C.** PGA group (Original magnification x 7500) **n:** nucleus, **v:** vacuole, **mi:** mitochondria, **mv:** microvillus, (*): biopolymer surface, one arrow (↑): attachment of spheroids to biopolymers. Unlike PGA groups, vacuolization in PLGA groups was rarely observed. Spheroids of all groups attached to biopolymers; at the attachment points, they lost their oval shape, took the shape of biopolymers' surface, became flat, and constituted a layer like tissue.

Taken together, according to ultrastructural evaluation, PGA and PLGA seemed to be more biocompatible with MDAH-2774 than other tumorigenic spheroids. Meanwhile, they were less biocompatible with SH-SY5Y spheroids and mouse calvaria primary osteoblast spheroids. The degree of biocompatility was greater for PLGA than for PGA in all spheroids.

DISCUSSION

We co-cultured PGA and PLGA with four tumorigenic (glioma, neuroblastoma, breast carcinoma, ovarian carcinoma) and two non-tumorigenic cells as well as their mature spheroids. The most resistant cell lines and their spheroids were determined to be human ovarian carcinoma MDAH-2774 and human osteoblast CRL-11372 for both PGA and PLGA. The most sensitive cell lines were human neuroblastoma SH-SH5Y and mouse calvaria primary osteoblast for these biopolymers. PLGA was found to be more biocompatible than PGA for all cell lines and their spheroids.

In this study, we used several complementary methods, such as Annexin-V-FITC/PI and TEM for the apoptotic index. Such approaches explained changes in the number of cells and BrDU-LI. BrDU-LI by immunohistochemistry only gave information about cells during the S-phase. The decreased number of cells could be due to arrests in the cell cycle at G0+G1 or G2+M. The effect of these biopolymers on the cell cycle should be tested using flow cytometric PI staining. Several molecular parameters related to apoptotic mechanisms (e.g., caspases) should also be tested to provide insights into the underlying mechanisms of the effects of these biopolymers. These recommendations can be defined as limitations in the current study, however our material and methods were convenient for explaining our hypothesis and testing the behavior of mature spheroids with PGA and PLGA.

Many studies aim to investigate synthetic biopolymers' biocompatibility by co-culturing single cells with these biopolymers over the long term to determine whether they are able to form spheroid or not. For example, Riccalton-Banks et al.[21] co-cultured rat hepatocytes with primary rat hepatic stellate cells on a biodegradable poly (DL-lactic acid) substratum. They found that these co-culture conditions encouraged rapid self-organization of three-dimensional spheroids that exhibited hepa-

tocyte-specific functionality (CYP-450 activity and albumin secretion) after almost two months in a static culture. Another example is Barralet et al.,[22] who investigated the behavior of primary human biliary epithelial cells (hBECs) on PGA and polycaprolactone (PCL)-stabilized PGA fiber mesh scaffolds. They found that cells formed spheroidal aggregates while continuing to proliferate in the long term and expressing phenotypic stability at PCL-stabilized PGA fiber mesh scaffolds. In both of these studies, cells formed spheroids with these biopolymers while the co-culture process continued over the long term. Spheroids became "mature" with these biopolymers. In the current study, mature spheroids were first formed and then incubated with PGA and PLGA. The difference was the manner of adaptation.

The connection of tumorigenic cells with biopolymers is commonly used in the era of chemotherapy to control the release of drugs for local delivery. Controlled release utilizes degradable or non-degradable polymers as carriers of chemotherapy; polymer implants or microparticles are implanted locally to introduce a sustained source of drug for periods of days or months.[23] They are also used after surgery as scaffolds, prostheses, and shunts.[1-4] After resecting tumors remnants, a single cell level can be left. Although filling processes using these biopolymers is done after chemotherapy, undesirable tumor recurrence can exist. Filling materials made up of these biopolymers can provide a niche for this recurrence. Thus, tumorigenic cell lines were also chosen in addition to non-tumorigenic cell lines in the present study. A few previous studies have examined tumorigenic cells' biocompatibility, but none were conducted at the mature spheroid level.

In the present study, significant changes in the number of cells were observed after 48th hour. The comparison of the resistance of tumorigenic cell lines ranked them in the order of MDAH 2774, MCF-7, SH-SY5Y and C6 glioma. Non-tumorigenic cell lines seemed more sensitive than tumorigenic cells; when compared among themselves, mouse calvaria primary osteoblasts seemed to be more resistant to changes in the environment. These data are in accordance with BrDU-LI results. S-phase cells increased in C6 glioma, MCF-7, and MDAH 2774. In contrast to the results related to the number of cells, BrDU-LI of human osteoblast was higher than mouse calvaria primary osteoblasts. In addition, the results related to the apoptotic index were consistent with BrDU-LI results. In this review, PLGA's apoptotic index was lower than PGA's for 96 hours of incubation for all groups. In contrast to BrDU-LI results, a high apoptotic index was seen in the PGA group of mouse calvaria primary osteoblasts. A high apoptotic index was seen in the PGA group of SH-SY5Y cells whereas a low apoptotic index was seen in the PGA group of MDAH 2774 among tumorigenic cell lines. High BrDU-LI and low apoptotic index can be the reason for significant tolerability of MDAH 2774 cells to these biopolymers.

The maintenance of immature or mature spheroid shapes means strong cell-to-cell adhesions and regular spheroid function.[24] In the present study, although we used biopolymers, spheroids derived from C6 glioma, SH-SY5Y neuroblastoma, and osteoblasts lost their specific shapes, surrounded the fibers, and filled the gaps between polymers. This process seemed to be similar to that of tissue constitution and can be interpreted as a decrease in cell-to-cell attachment within the spheroid. Instead of forming independent aggregate or spheroid and/or spreading around the medium, they chose to attach to biodegradable polymers. The invading manners of C6 glioma, SH-SY5Y neuroblastoma, and osteoblast spheroids were also noteworthy. If there was only one PGA or PLGA polymer fiber was in the medium, the spheroids took fibers inside like phagocytosis and arranged them in an order like polysomes without losing their shapes. If two PGA or PLGA polymers were in the medium, they lost their specific shape, invaded through polymers, and filled the gaps between polymers. In accordance with these synergistic morphologic results, subtle decreases in the ratio of S-phase tumorigenic and non-tumorigenic spheroids co-cultured with polymers strengthened this synergism.

The reduction rates of BrDU-LI in spheroids were almost the same among tumorigenic groups

for PLGA; only the reduction rates of MCF-7 were lower than others at PGA. The reduction rates of PGA were more than PLGA in the CRL 11372 cell line, but the reduction rates for both biopolymers were almost same in MCPO. However, the results of BrDU-LI provided from monolayer cultures revealed differences among tumorigenic cell lines and among non-tumorigenic cell lines. Mature spheroids seemed to be more resistant to the effects of biopolymers than a single cell.

The ultrastructure of spheroids with biopolymers was similar to the control group except for the presence of increased vacuoles and rarely seen apoptotic evidence-another result that strengthens this synergism. The vacuolization occurred more often in PGA-incubated spheroids. All spheroids chose to attach to biopolymers rather than to form clusters with each other. Although mature spheroids possessed huge necrotic and apoptotic zones as a result of their age, their affinity to biopolymers seemed to be similar to the affinity of young spheroids.

Cancer cells are transformed cells that challenged apoptosis to survive.[9] When they are compared to non-tumorigenic cell lines, their response to acidification varies with the origin and type of cell line. Previous studies demonstrated that PGA and PLGA degraded primarily through the simple hydrolysis of ester bond into acidic monomers, which can be removed from the body via normal metabolic pathways. Hydrophobicity and molecular weight both affect degradation. The degradation of a block co-polymer PLGA is affected by the ratio of hydrophilic PGA to hydrophobic poly lactic acid (PLA).[25] Sung et al.[26] conducted a comparative study of quickly (PLGA, 50:50) versus slowly degrading (poly ε-caprolactone, PCL) polymers, indicating that quick degradation negatively affected cell viability and migration of scaffolding both *in vivo* and *in vitro*. They explained this effect based on the significant acidification of the local environment due to polymer degradation. Korkmaz et al.[27] further showed that, among the tested polymers on monolayer cultured cells and three-dimensional spheroids, PLGA was more biocompatible than PGA. Consistent with these studies, PLGA performed best with respect to cell attachment, invasive behavior, and active mitotic activity in the present study.

Compared to PLGA, the adaptation of cells and spheroids to PGA seemed to take more time; their adaptation ratio also increased in a time-dependent manner. Sensitivity to PGA became clear at the C6 glioma and osteoblast cell lines. Di Toro et al.[28] demonstrated that the initial adhesion of osteoblasts on implant surfaces required the contribution of integrins, acting as a primary mechanism regulating cell-extracellular matrix (ECM) interactions. They observed that adhesion of osteoblasts to PLA-PGA copolymers two hours after plating was reduced by approximately 70% by antibodies capable of blocking integrin beta(1) and alpha(5)beta(1) complexes but by only 30% by an anti-integrin alpha(v) antibody. Therefore, the authors suggested that beta(1) integrins may represent a predominant adhesion receptor subfamily utilized by osteoblasts to adhere to PLA-PGA copolymers. Similar to this study, the crosstalk of integrins and PLGA can be the result of high biocompatibility of both tumorigenic and non-tumorigenic cell lines to PLGA.

CONCLUSION

In conclusion, the present study provides a detailed characterization of the *in vitro* biocompatibility of PGA and PLGA on mature spheroids of tumorigenic and non-tumorigenic cell lines. PGA and PLGA were biocompatible with these mature tissue-like structures, although PLGA was more compatible than PGA. The characterization of cell-biopolymer interactions and resistance levels at the increasing age of multicellular spheroids can provide data for optimizing the use of these biopolymers and their risks in the treatment of older patients and older cancer patients. Thus, these results may shed light on neglected reasons for treatment failures.

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