Benchmarks

Laser Capture Microdissection in 2-D Co-Culture Models as a Tool to Study Tumor-Stroma Interactions

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Cellular adhesion, migration, and invasion are essential processes in tumor progression (5). These processes include actions by tumor cells as well as by the stromal microenvironment. In the last few years, it has become evident that tumor cell behavior and progression are affected by tumor stroma, including peritumoral fibroblasts (4,8). In vitro, tumor cell-stromal fibroblast interaction can be studied by 2-D and 3-D co-culture models (9). In conventional 2-D co-culture systems, the different cell types are grown together in glass-slide-based chambers with medium. Immunohistochemical detection at the protein level can be easily performed; however, for protein and gene expression analysis, the cell compartments have to be dissociated and sorted, for instance, by flow cytometry or magnetic cell separation. These procedures could possibly result in stress-induced alterations in RNA expression.

Here we present a novel technique for co-culturing and separating fibroblasts and carcinoma cells. It is based on cell co-cultivation on a 1.35- μm -thin membrane combined with laser capture microdissection (LCM) of the cell compartments after rapid immuno-

labeling. LCM permits the rapid and reliable procurement of populations of cells from tissue sections by infrared laser beam power, resulting in samples that are well suited for subsequent molecular analysis (2,6).

We show that our technique of co-culture-LCM allows the extraction of intact RNA from a limited number of co-cultivated cells separated without cell cross contamination after immunostaining as a tool for expression profiling and genetic analysis.

The tumor cell line PE/CA-PJ34 (ECACC accession no. 97062513), as a model of an invasive tumor, was established in our laboratory from a surgically removed squamous cell carcinoma of the oral cavity as described previously (1). Tumor differentiation and matrix protein expression in this cell line were characterized immunohistochemically after culturing on 4-well cell culture slides (FALCON®/CBP; BD Biosciences, Heidelberg, Germany). We used the human fibroblast cell line hTERT-BJ1 (BD Biosciences Clontech) as an artificial stroma compartment.

hTERT-BJ1 fibroblasts (5×10^4 ; Invitrogen GmbH, Karlsruhe, Germany) were allowed to adhere to 2×2 cm membrane fragments floating in cell culture medium (IMDM, 10% FCS, and 80 $\mu\text{g}/\text{mL}$ gentamycin). This special 1.35- μm -thin membrane (P.A.L.M. GmbH, Bernried, Germany) was originally developed for the mounting of histological tissue sections on regular glass slides for the so-called MicroBeam-MOMeNT (microdissection of membrane mounted native tissues) technique, mainly to hold the microdissected area

or specimen together during the catapulting procedure with the PALM LCM System (P.A.L.M. GmbH). The fibroblasts were cultured until subconfluent on the upper side of the membrane, subsequently overlaid with 5×10^4 carcinoma cells and co-cultured for 6–10 days.

The membrane does not influence cell growth. Differences in growth behavior, cell morphology, and laminin-5 immunostaining between cells cultivated on slides and on the membrane were not observed (data not shown).

The co-culture membranes were fixed in 70% ethanol for 1 min and transferred to RNase-free glass slides. After flattening and air-drying, the membranes were fixed with rubber cement. For identifying the tumor cell compartment, immunolabeling for the laminin $\gamma 2$ chain was performed, a marker that is expressed only in epithelial tumor cells. We used a modified immunostaining protocol based on the ChemMate™ Detection Kit AP/red (Dako A/S, Glostrup, Denmark) that allows the identification of the tumor cells and maintains the DNA and RNA integrity for subsequent analysis. Membranes were incubated for 10 min at room temperature with the anti- $\gamma 2$ chain antibody GB3 (Harlan Sera-Lab Ltd., Leicestershire, UK) at a concentration of 60 $\mu\text{g}/\text{mL}$ diluted in Antibody Diluent Solution (ZYMED® Laboratories, San Francisco, CA, USA) supplemented with 1 U/ μL RNase inhibitor (Roche Applied Science, Mannheim, Germany). After rinsing three times in RNase-free PBS, detection of the primary antibody was performed using the ChemMate Detection Kit AP/red according to the

Figure 1. Co-culture-LCM. Panel a represents a survey of the 2-D membrane co-culture model. The tumor cell areas are immunostained with the laminin $\gamma 2$ chain antibody GB3 (arrows). For better visualization, fibroblasts are counterstained with hematoxylin (bar = 100 μm). Immunostained tumor cell areas (b) or unstained fibroblasts (c) are microdissected with the laser beam. The laser tracks are clearly visible (arrowheads) and the precision of the procedure enables the dissection of the area of interest without cross contamination (bars = 100 μm).

Figure 2. Amplifiable cDNA obtained after LCM of co-cultivated tumor cells and fibroblasts. The 326-bp cyclophilin fragment (arrow) is seen after RT-PCR in tumor cells of the PE/CA-PJ34 cell line (lane 1) and hTERT-BJ1 fibroblasts (lane 2), both referring as controls as well as in immunostained microdissected co-cultivated tumor cells (lane 3) and fibroblasts (lane 4). No cyclophilin amplification can be detected if reverse transcriptase is omitted in RT-PCR (no-RT-control), indicating that the RNA from microdissected tumor cells is free of contaminating DNA (lane 5). The laminin $\gamma 2$ chain cDNA (460-bp fragment, arrow) is amplifiable only in tumor cells from co-cultivation (lane 6) and not in the co-cultivated fibroblasts (lane 7), indicating no cell cross contamination during microdissection.

manufacturer's instructions. The incubation time of the secondary biotinylated antibody and the alkaline phosphatase-conjugated streptavidin inhibitor was shortened to 5 min at room temperature. The color development was monitored microscopically. All reagents were supplemented with 1 U/ μ L RNase inhibitor. After rinsing in DEPC-treated water, membranes were sequentially dehydrated in 75%-95%-100% ethanol and subsequently dried in a fume hood. For counterstaining, a short protocol using hematoxylin can be used but is not mandatory for visualization.

After identifying tumor cells by laminin $\gamma 2$ chain immunostaining, about 5×10^3 each labeled (tumor) and unlabeled (fibroblast) cells were microdissected with the PALM LCM System and captured in denaturation buffer from the Micro RNA Isolation KitTM (Stratagene, La Jolla, CA, USA). The amount of starting material depends on the cell type and the RNA transcripts of interest for downstream applications. Previously, we were able to show that $3\text{--}5 \times 10^3$ cells are sufficient for successful total RNA extraction and in vitro amplification for gene expression analysis on microarrays (3).

As shown in Figure 1, tumor cells and fibroblasts could be easily distinguished and microdissected without cross-contamination.

Isolation of amplifiable DNA from

the microdissected cells can be performed with standard procedures (data not shown). Analysis of RNA molecules has been more troublesome and can be a major limiting step for downstream applications because of loss of RNA quality and quantity during RNA sample preparation from a limited number of immunostained cells. Previously, we developed a total RNA extraction protocol for limited amounts of starting material that combines a guanidinium thiocyanate-based denaturation with a modified RNA precipitation and purification (3). This protocol also worked perfectly under the immunostaining conditions described here.

After total RNA extraction, reverse transcription with 1 μ L 25 μ M Oligo (dT)₁₅ primer was performed using the SUPERSCRIPTTM Choice System according to the manufacturer's guidelines (Invitrogen GmbH). For each sample, a control reaction was performed without the addition of reverse transcriptase to prove the absence of amplifiable DNA in the RNA extractions. cDNA amplification was carried out with 30–40 amplification cycles with primers (ams biotechnology GmbH, Wiesbaden, Germany) for the housekeeping gene transcript cyclophilin and for the laminin $\gamma 2$ chain as a marker for epithelial tumor cells (7). Eight microliters of amplification product were run on a 2% agarose gel. We examined the RNA quality from the microdissected co-cultured cells by RT-PCR for a housekeeping gene transcript and for the laminin $\gamma 2$ chain gene transcript used in immunostaining the tumor cell compartment (Figure 2). We show in this study that laser capture-microdissected tumor and stromal cells from the membrane-based co-culture model can be used for gene expression profiling and DNA based analysis.

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