Cancer research with sole focus on the cancer cell and possibly growth factors cannot faithfully reproduce the environmental interaction, such as adhesion of tumor cells to e.g. stromal cells, which may determine the response of these tumors to therapy. Methodologically cell adhesion studies are often difficult since complete but careful detachment is the prerequisite for most signal transduction assays. We describe for the first time an alternative method for the co-incubation of multiple myeloma cells on long term primary bone marrow stromal cultures using the bone marrow stromal cell line HS-5. The methods are precisely described, advantages and disadvantages are discussed, and troubleshooting advises are given.

**Key Words:** cell adhesion, cell-cell contact, methodology, stromal cells, myeloma cells.

**MATERIALS AND METHODS**

Cells were grown in RPMI 1640 medium (Boehringer, Germany) containing 10% heat — inactivated fetal calf serum (FCS; Boehringer) in a humidified atmosphere (37.5 °C; 5% CO₂).

**Primary bone marrow stromal cells.** The generation of a confluent layer of primary bone marrow stromal cells was performed according to the protocol of Lokhorst and colleagues [5, 6]. Mononuclear cells were separated from bone marrow aspirates by Ficoll-Paque (Amersham Pharmacia, Sweden) using LeucoSep (Greiner, Germany) tubes. Bone marrow mononuclear cells (BMMC) were washed twice with 2% FCS-PBS and seeded at 2 x 10^6 cells per millilitre in plastic flasks or well plates using medium with 20% FCS. After one week half of the supernatant was discarded and renewed with fresh medium. The procedure was repeated weekly until a confluent layer of stromal cells was grown. In average it took about 6–9 weeks.

After this time complete medium was removed weekly to clear the culture from remaining round cells. In our experience the stromal culture could be split using trypsin two times to expand the material. Most cultures showed no further growth after the third passage.

**Co-culture of primary bone marrow stromal cells and multiple myeloma cells.** Multiple myeloma cells (NCI-H929, OPM-2, U266, RPMI-8226 cell lines) were seeded at 10^6/ml on the stromal cell layer. Adherence was observed after 15 to 30 minutes. Longer co-incubation did not further increase adherence. Therefore, 1 h after myeloma cells were seeded cytotoxic compounds, like melphalan, treosulfan, bortezomib, dexmethasone, or doxorubicine, were added. After 48 to 72 h myeloma cells were detached by pipetting
vigorously using a 1000 µl pipette. All myeloma cells should be removed (the firmly adherent cells are of major interest). This has to be checked by microscopy. On the other hand, stromal cells may not be detached because these very large cells cause clumps and make further processing of the material impossible. Avoid use of cell scrapers or trypsin, as detached cells should be intact and alive. Staining with trypan blue checked cell shape and membrane integrity.

Co-cultures with primary human bone marrow stromal cells have several disadvantages: firstly, the availability of bone marrow aspirates is limited, even at specialized centres. The procedure of bone marrow aspiration is painful and therefore ethical approval and patient’s consent must be obtained. Many patients have been treated with cytotoxic compounds and almost all donors have hematological (bone marrow) disease. There is increasing evidence that bone marrow stromal cells are crucially involved in the pathophysiology of these diseases and therefore may display an altered phenotype compared to “normal” stromal cells. Additionally, a single bone marrow probe allows performance of a very little number of experiments. Therefore, investigations on myeloma cells have to be performed using stromal cells from different donors. Thus, comparability of experimental series is significantly hampered. Secondly, it takes several weeks until a confluent layer is grown with a high risk of bacterial contamination and herewith loss of the valuable stromal cell sample.

Thirdly, the mechanical detachment of myeloma cells from the stromal layer by pipetting and scratch- ing injures the cells and e.g. increases the annexin V positivity and herewith falsifies the results. Additionally, signal transduction research on primary stromal cells itself is very difficult due to the low availability as well as the large cell volume that interferes with flow cytometry analyses.

We attempted to facilitate the work with primary bone marrow stromal cells and have therefore established a protocol using the bone marrow stromal cell line HS-5. This method provides easy handling, good reproducibility, and low risk of false results.

**Co-culture of HS-5 bone marrow stromal cells and multiple myeloma cells.** HS-5 cells were obtained from the American Type Culture Collection (Rockville, USA). Three days before the co-culture HS-5 cells were trypsinized in the culture flask with 8 ml Trypsin (Trypsin-EDTA, Sigma, Germany), washed twice, and then resuspended in 20 ml RPMI-1640/10%FCS. HS-5 were seeded in 6-, 12- or 24-well plates at a concentration of 5x10^4/ml for 3 days until a confluent layer has developed. Multiple myeloma cells (NCI-H929, RPMI-8226, OPM-2 and U266) were added to the HS-5 layer at a concentra- tion of 1.5x10^5/ml for a two-day incubation period or at 10^5/ml for incubation over 4 days. After adhesion (30 min) cytotoxic drugs and pathway modulators were added. On day 2 (or 4), co-cultures were checked by microscopy (Fig 1, a). All cells were detached from the well using a cell scraper, which can be very easily and gently done. This procedure does not affect cell shape (microscopy), membrane integrity (propidium iodide, trypan blue) and asymmetry of the membrane (annexin V). Myeloma and stromal cells are suspended in medium and can be further processed for biological or signal transduction assays. For example, Fig. 1, b, shows that co-cultured cells can be easily separated by flow cytometry after CD38-FITC staining. Using a second or third fluorescent, characteristics of the myeloma cells can be determined. Here cell death upon melphalan treatment determined by propidium iodide uptake is shown (see Fig.1, b). Furthermore, monoculture of myeloma cells may be compared to co-culture regarding melphalan induced cell death. Fig. 1, c, demonstrates that co-cultured myeloma cells are much less susceptible to melphalan. Additionally, myeloma cells can be purified by magnetic cell sorting (anti-CD138-MACS, Miltenyi Biotec, Germany) and expression of pathway proteins can be determined by western blotting, as described [7]. Fig. 1, d, shows unaltered protein expression levels of antiapoptotic proteins (Bcl-2, Bcl-XL) and important signalling kinases (Erk-2, Akt, PKCα). For further results see [4]. Of note, we have shown that — at least regarding cell adhesion mediated drug resistance — HS-5 cells display comparable results with primary bone marrow stromal cells [4].

**DISCUSSION AND TROUBLE SHOOTING**

An important question was whether apoptotic/necrotic cells still express sufficient amounts of surface antigens to ensure proper separation of myeloma from the bone marrow stromal HS-5 cells. For this reason we always performed control experiments with myeloma cell monocultures to exclude a possible CD38 negative second myeloma population. Fig. 2, a and 2, b, demonstrate that at higher concentrations of cytotoxic compounds expression levels of certain surface antigens like CD38 are lowered. Even at high concentrations, however, myeloma cells do not completely lose a detectable CD38 expression, thus no CD38 negative second population of dead myeloma cells could be misinterpreted as HS-5 cells.

Furthermore, despite a certain reduction of surface expression of CD38, myeloma cells can be clearly separated from HS-5 stromal cells. Interestingly, some necrotic myeloma cells just resolve, since the ratio between myeloma and stromal cells declines upon chemotherapy treatment (Fig. 2, c). However, these cells do not perturb our assay. Occasionally, myeloma cells do not adhere to HS-5 cells properly. This is due to a false concentration of myeloma cells (above 2 x 10^5/ml) or a too extensive use of trypsin. One limitation of this model is the fact that HS-5 are quickly proliferating cells (doubling time 72 h). Experiments, which depend on incubation periods longer than four days are difficult to perform. Primary seeding concentration has to be reduced in such cases, but below a certain threshold adhesion is no longer guaranteed. In ac-
cordance in some experiments more myeloma cells were PI positive in the coculture control than in the monoculture control. This was the result of inadequate HS-5 cell numbers. Because HS-5 cells turn medium acidic very quickly, their number should not be too high. This depends on the used culture flask but in general the amount of HS-5 cells may never exceed the space they have to adhere.

Outside-in signaling is not only mediated by soluble ligands, but as well by adhesion molecules that mediate direct interaction with other cells. Cancer cells are characterized by certain organ preferences in terms of primary growth and as well in terms of metastases.

Circulating tumor cells anchor to, survive and proliferate in these target organs whereas other organs are spared. Convincing examples are the adrenal gland metastases of the — neuroendocrine differentiated — small cell lung cancer, or the diffuse infiltration of the entire skeleton by multiple myeloma cells sparing almost all other tissues. Cell-cell contact mediated signalling research depends on coculture experiments. We have described the frequently used method of primary long term bone marrow stromal cell culture, because in most publications important details are not given, which are necessary for reproduction. This method is limited by the large size of the stromal cells and difficulties in detachment, the prerequisite for further signalling research. We therefore have developed a method using immortalized human bone marrow stromal cells, which has many practical advantages. Due to the high proliferation rate of the HS-5 cells this method displays certain limitations.

**Fig. 1.** Co-culture of HS-5 bone marrow stromal cells and NCI-H929 multiple myeloma cells. 

a. Microscopic evaluation (original magnification x 100, unstained) of confluent primary bone marrow stromal cells (*) and adherent NCI-H929 myeloma cells (°) after 48 h treatment with 20 µM melphalan. # indicates a non viable myeloma cell. 

b. Separation of NCI-H929 myeloma cells (CD38+) and bone marrow stromal cells (CD38-) by flow cytometry. Propidium iodide (PI) uptake in gated myeloma cells after 48 h of 20 µM melphalan was determined. 

c. Determination of cell adhesion mediated drug resistance. 

**CD38+ myeloma** cells were gated after 48 h of 30 µM melphalan in monoculture (NCI-H929) or co-culture (NCI-H929/HS-5). Percent of propidium iodide (PI) positive cells was determined by flow cytometry. 

d. CD38+ NCI-H929 were purified by magnetic cell sorter after monoculture (NCI-H929) or co-culture (NCI-H929). Expression levels of antiapoptotic proteins and signalling kinases were determined by western blotting.
as well, but with the described protocol reproducible and reliable experiments can be performed easily and quickly. As we have previously shown [4] that the biological effects induced by stromal cell adhesion are similar for primary stromal cells and HS-5 cells, both methods can be used alternatively depending on the special experimental requirements.

ACKNOWLEDGMENTS:

This work was supported by the Förderprogramm für Forschung und Lehre (FöFoLe) der Universität München (LMU), grant no. 271 (to R.S.).

REFERENCES


Fig. 2. Stable expression of surface marker CD38 on myeloma cells after melphalan treatment. NCI-H929 myeloma cells were treated for 48 h with 20 µM (a) or 30 µM (b) melphalan in monoculture (NCI-H929) or co-culture (NCI-H929/HS-5) and CD38 expression was determined by flow cytometry. c. NCI-H929 myeloma cells were treated with 20 µM melphalan in the presence of HS-5 stromal cells. The relative CD38+ population is decreased in comparison with untreated control