Morphological heterogeneity and phenotypical instability versus metastatic stability in the murine tumor model ER 15-P

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Summary. At clinical presentation, the majority of malignant tumors are composed of multiple clonal subpopulations of tumor cells with different phenotypic characteristics. Using the experimental tumor model ER 15-P, a methylcholanthrene-induced pleomorphic sarcoma of the C57 Bl/J mouse, we studied a system of long-term in vivo passages of this primary tumor for cell morphological changes, and alterations in the potential for spontaneous lung metastases. Transplants from the primary after the 4th, 20th, 40th and 80th i.m. passage (referred to as T4, T20, T40, and T80 respectively) together with their lung metastases were investigated by light microscopy, immunohistochemistry, and electron microscopy. In addition, the potential for metastasis to the lungs in each group was determined and compared with that of the parent T4 tumors. T4 tumors were mainly composed of spindle-shaped tumor cells with the ultrastructural features of fibroblasts and myofibroblasts, often arranged in a storiform or fasciculated growth pattern, and intermingled with tumor giant cells. Some small areas contained polygonal or rounded tumor cells, ultrastructurally undifferentiated, and sometimes arranged in a hemangiopericytoma-like growth pattern. Although electron-microscopical findings clearly demonstrated the mesenchymal origin of these tumor cells, immunostaining with a polyclonal antibody to vimentin was unspecific in all tumor cells and normal mouse tissue. Monoclonal antibodies to vimentin from different sources were completely negative in tumor cells and murine stromal components. In contrast, myofibroblast-like tumor cells showed immunohistochemically, a moderate to strong co-expression with monoclonal antibodies to desmin, muscle actin and α-smooth muscle actin. On the basis of these morphological findings, the primary ER 15-P was classified as a pleomorphic myofibrosarcoma. The lung metastases of T4 tumors were mainly composed of undifferentiated round to polygonal tumor cells, while the number of desmin-positive, muscle- and α-smooth muscle-actin-positive cells was reduced. The morphological features of T20 tumors and their lung metastases were the same as in T4, indicating a relative stability of the phenotype up to that stage. In contrast, T40 and T80 tumors and their lung metastases were found to contain almost exclusively undifferentiated tumor cells and many tumor giant cells. While fibroblast-like tumor cells were seen only occasionally, myofibroblast-like tumor cells had almost completely disappeared. The potential for lung metastases was nearly constant in all groups, suggesting metastatic stability. Obviously, the undifferentiated tumor cells of this model are associated with a higher metastatic potential.

Key words: Pleomorphic myofibrosarcoma ER 15-P – Morphological heterogeneity – Lung metastases – Metastatic potential

Introduction

Cellular heterogeneity, i.e. the coexistence of multiple clonal tumor cell subpopulations within an individual neoplasm, is recognized as a fundamental phenomenon in malignant tumors (Fidler and Hart 1982; Heppner 1984; Welch and Tomasovic 1985; Nicolson 1987; Heppner and Miller 1989; Killion and Fidler 1989). It is currently accepted that such heterogeneity results from sequential cellular changes occurring during tumor growth, and ultimately leading to the selection of new cell variants with quite different characteristics (Cairns 1981; Klein and Klein 1985; Nicolson 1987; Nowell 1976, 1989). The process appears to be independent of the actual monoclonality of a malignant tumor (Iannaccone et al. 1987), and to be influenced by genetic and epigenetic mechanisms such as host environmental factors, and interactions between tumor and normal host.
cells (Nicolson 1987; Marcel et al. 1990; Miller and Heppner 1990; Rubin 1990).

It is important that heterogeneity does exist in a malignant neoplasm from its earliest detection prior to any influence of treatment (Heppner and Miller 1989). Heterogeneity manifests itself in a wide spectrum of tumor cell properties, of which the capacity for invasion and metastatic spread, the propensity for developing genetic variants, and the differential sensitivity to chemotherapy are the clinically most important features (Nicolson 1987; Heppner and Miller 1989; Killion and Fidler 1989; Nowell 1989; Fidler 1991). Pathologists have been aware for quite some time that malignant tumors contain multiple cell populations of distinctly different morphological features (Willis 1973; Katenkamp 1988). In a growing tumor, however, a precarious equilibrium seems to exist between tumor subpopulations and host cells, ensuring the relative stability of the histological phenotype over a certain period (Baylin and Mendelsohn 1982). Experimental data indicate that the very coexistence of certain subpopulations in a polyclonal tumor, may serve to mediate phenotypic diversification, and to restrict the emergence of cell variants (Miller et al. 1980, 1981; Poste et al. 1981; Miner et al. 1982; Itaya et al. 1989; Heppner 1989). The disturbance of the equilibrium between tumor cell subpopulations by various selection pressures in vivo or in vitro may eventually lead to the emergence of new variants with differing phenotypes (Poste et al. 1981; Heppner 1984; Nicolson 1987; Heppner and Miller 1989). So far, only a few studies have investigated the influence of long-term in vivo transplantations of primary tumors into syngeneic rodents, on cell morphology and metastatic potential (Isaacs 1982; Isaacs et al. 1982; Barnett and Eccles 1984).

Starting from these data we wanted to find out whether long-term in vivo passaging of tumor cells in the murine tumor model ER 15-P leads to alterations of phenotype in terms of cell morphology and metastatic potential. The study was performed with light microscopy, immunohistology and electron microscopy in a spontaneous metastasis assay, i.e. using in vivo intramuscular transplantation of tumor cells in animals.

Materials and methods

Animals. Female C57 Bl/J mice aged 6–8 weeks, obtained from Zentralinstitut für Versuchstiere, Hannover, were kept individually in Macrolon cages on Altromin littering, and fed an Altromin standard diet with water ad lib. Room temperature was maintained at 21 ± 2°C. Relative humidity at 60 ± 5%.

Tumor cells. The assay was performed with the syngeneic, spontaneously metastasizing, pleomorphic murine sarcoma ER 15-P, originally induced by methylcholanthrene in a female C57Bl/J mouse (Edel 1984; Edel and Grundmann 1984). The primary is weakly immunogenic (Edel 1983). From that tumor, a cell line with liver-specific metastatic potential (ER 15-M) had been isolated previously (Edel 1988; Edel and Grundmann 1984). The liver-specific spread of these tumor cells was based on the selection of a tumor cell variant from the primary that was preferentially influenced by the hepatic environment (Edel 1988a, b).

Preparation of tumor cells. Samples of 4 × 10⁶ tumor cells taken from the whole tumor mass of the 4th, 20th, 40th, and 80th transplant generation of the primary ER 15-P (referred to as T4, T20, T40, and T80 in the following text) were frozen in RPMI-1640 medium, supplemented with 0.038% NaHCO₃, 0.005 M l-glutamine, 0.05 M HEPES buffer, penicillin (50 units/ml), streptomycin (50 μg/ml), fungizone (2.5 μg/ml) (all from Gibco, BRL Eggengstein, Leopoldshafen, FRG), 10% dimethylsulfoxide (SERVA, Heidelberg, FRG) and 40% heat-inactivated fetal calf serum (Gibco), and then stored in liquid nitrogen. The tumor cells were thawed rapidly in a 37°C water bath, and resuspended with the above culture medium containing 10% fetal calf serum without dimethylsulfoxide. They were washed three times in medium by centrifugation for 10 min at 120 g/min, and ultimately resuspended in wash medium composed of Hank’s balanced salts solution with double-distilled water 1:10, with the addition of 0.005 M l-glutamine, 0.038 NaHCO₃, and 0.05 M HEPES buffer (all from Gibco). Tumor cell viability was determined by trypan blue exclusion. All suspensions were found to contain 50%–60% of the original tumor cell concentration. Samples containing 1 × 10⁶ tumor cells from T4, T20, T40, and T80 were injected individually in the left femoral muscle of mice. Two weeks later, when the i.m. growing tumors had reached a maximum diameter of about 1.5 cm, the animals were killed by ether anesthesia. To avoid artificial segregation of tumor cell subpopulations, as may occur occasionally by transplantation of pieces or portions of tumor tissue (Fidler and Hart 1981), a suspension was prepared from the whole tumor of each transplant generation, using a combined mechanical and enzymatic method described previously (Edel 1988a).

The viability of the resulting cell suspension was about 60%–80% as determined by trypan blue exclusion.

Experimental design. Samples of 1 × 10⁵ tumor cells from T4, T20, T40, and T80, respectively, were injected into the left femoral muscles of ten mice per group. The animals were kept separately and monitored individually for the beginning of tumor growth. After 3–4 weeks, when tumors of about 3 cm maximum diameter had developed, the mice were killed as mentioned above. The primary, the lungs, and all other organs including lymph nodes of each animal in the respective groups were carefully resected. Samples of the primaries and lungs from each group were prepared either for light and immunohistological investigation, or for electron microscopy (see below). Eight lungs of each group were fixed in Bouin’s solution for quantification of metastases as described previously (Edel and Grundmann 1984). The other organs were inspected macroscopically for evidence of metastatic tumor growth.

Light and immunohistochemical microscopy. Samples of the primaries from each group were fixed either in Bouin’s solution or in 4% phosphate-buffered formaldehyde for 24 h and primaries and lungs were embedded in paraffin. For light microscopy, 3- to 4-μm sections were stained with hematoxylin/eosin (H&E), periodic acid/Schiff (PAS), elastica/van Gieson, Gomori, phosphotungstic acid/hematoxylin, and Masson trichrome. Immunohistochemistry was also performed on paraffin sections of these tumor samples.

The primary polyclonal and monoclonal antibodies used are listed in Table 1. All primary antibodies were diluted in a modified RPMI-1640 medium (Sigma, Deisenhofen, FRG) containing 5 ml RPMI-1640 medium, 45 ml double-distilled water, 5 ml heat-inactivated bovine serum (Sigma) and 0.05 g sodium azide (pH 7.4–7.6). A three-step alkaline phosphatase method was used for polyclonal antibodies: the sections were mounted on egg-white-coated slides and left to dry overnight at 37°C. They were then deparaffinized and rehydrated in graded ethanol. The slides were incubated in a moist chamber at room temperature for 45 min with the respective primary antibody. After washing in 0.05 M TRIS/NaCl buffer, the sections were each treated for 30 min with the appropriate secondary antibody, either goat-anti-rabbit IgG or rabbit-anti-goat IgG conjugated with alkaline phosphatase (Dianova, Hamburg, FRG), and the tertiary antibody either rabbit-anti-goat IgG or goat-anti-rabbit IgG conjugated with alkaline phosphatase (Dianova),...