MALIGNANT MESOTHELIOMA, CLINICAL, DIAGNOSTIC AND CELL BIOLOGICAL INVESTIGATIONS

Het maligne mesothelioom, klinisch, diagnostisch en celbiologisch onderzoek

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. C.J. Rijnvos
en volgens besluit van het college van Dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 22 november 1989 om 13.45 uur.

door

Marjan Amelie Versnel
geboren te Utrecht

1989
Offsetdrukkerij Haveka B.V.,
Alblasserdam

Promotiecommissie

Promotoren	: Prof. Dr. R. Benner Prof. Dr. A. Hagemeijer
Overige leden	: Prof. Dr. D. Bootsma Prof. Dr. E.J.J. van Zoelen

Dit proefschrift werd bewerkt binnen de afdeling Celbiologie, Immunologie en Genetica van de Erasmus Universiteit Rotterdam.
Het onderzoek werd mogelijk gemaakt door financiële steun van de Nederlandse Kankerbestrijding (Stichting Koningin Wilhelmina Fonds).
Deze publikatie werd mede mogelijk gemaakt door Haagman Advocaten te Rotterdam.

Aan mijn ouders
Aan Hans en Frank

CONTENTS

PREFACE

Cancer is the unrestrained proliferation of malignant cells. It is now generally thought that the basis of cancer is the damage of genetic material. This damage can have different causes like radiation and chemical agents. In many types of cancer damage of genetic material has been demonstrated in the form of aberrations of the chromosomes and/or DNA. These aberrations have been found to be specific for different tumor types and are in many cases of diagnostic significance.

Investigations towards the unlimited growth potential of tumor cells *in vitro* revealed that these cells are independent of exogenous growth factors. This independence and the unrestrained proliferation of tumor cells *in vivo* suggested that growth factors and growth regulation of normal and malignant cells was next to the genetic studies an important characteristic to be approached in cancer research.

Human malignant mesothelioma is a tumor that is most frequently found in the covering of the chest cavity, called the pleura. The incidence of malignant mesothelioma is strongly associated with asbestos exposure. In the areas of Rotterdam and Vlissingen the incidence of malignant mesothelioma is increased compared to other parts of the Netherlands. This is caused by the extensive use of asbestos in the shipbuilding and repair industries in these areas in the past. Diagnosis of malignant mesothelioma on pleural effusion cells is difficult since there are no morphologic criteria for differentiation between benign and malignant mesothelial cells and adenocarcinoma cells. Establishment of a definite diagnosis on pleural effusions is important as it prevents more invasive diagnostic procedures like the taking of a biopsy for histological examination.

The purpose of this study was to improve the diagnosis of human malignant mesothelioma on the one hand and to investigate the growth regulation and transformation of normal and malignant mesothelial cells on the other hand.

In this thesis improvement of diagnosis was approached by the search for specific tumor markers. Therefore malignant mesotheliomas were analysed for their chromosomal aberrations and the expression of specific membrane antigen markers. For the investigation of the growth regulation in normal and malignant mesothelial cells a panel of *in vitro* growing malignant mesothelioma cell lines was established. These cell lines were studied for expression of growth factors and their receptors, with

emphasis on the platelet-derived growth factor and its receptor. Evidence has been obtained that platelet-derived growth factor (PDGF) may play a role in the malignant transformation of mesothelial cells.

CHAPTER 1

HUMAN MALIGNANT MESOTHELIOMA

1.1 Epidemiology

Malignant mesothelioma is a tumor derived from the mesodermal tissues of the coelomic cavities. Mesotheliomas occur in the pleura and less frequently the peritoneum, pericard and tunica vaginalis testis. In 1960 Wagner et al. described the occurrence of malignant mesotheliomas among workers in South-African asbestos mines. Other epidemiological reports confirmed the relationship between asbestos exposure and malignant mesothelioma (McDonalds et al., 1977). In approximately 85% of the malignant mesotheliomas exposure to asbestos could be traced. The latency period between asbestos exposure and the occurrence of a malignant mesothelioma ranges from 15 to over 40 years. Due to the occupational exposition approximately 70% of the malignant mesothelioma patients are men.

The incidence of malignant mesothelioma in the developed countries is still increasing. According to the use of asbestos in the past, the long latency period and the fact that control measures have been taken since the seventies only, it has to be expected that the incidence of malignant mesothelioma will increase at least until the year 2000. A recent projection for asbestos related malignant mesothelioma mortality in the United States for the period 1985 - 2009 was estimated to be 21,500 (Lilienfeld et al., 1988). In the Netherlands 200 - 300 cases are diagnosed yearly and an increase until 500 has to be expected for in the year 2000.

1.2 Asbestos and malignant mesothelioma

Asbestos is a family of silicate minerals which includes chrysotile, crocidolite and amosite as the industrially most significant fibers. The thermal, chemical and physical properties of these fibers are the cause of their technological importance. Asbestos was and still is used in e.g. shipbuilding industries, flooring products, insulation, and brake lining. Inhalation of crushed asbestos fibers is the major health risk in these industries. The carcinogenic effect of asbestos fibers has been found to be dependent on the physical properties of the fibers. Respirable fibers with a diameter less than 0.1 μm and a length over 5.0 μm were found to be responsible for mesotheliomas, but other sizes are also mentioned (Lippmann, 1988). Asbestos also can cause benign pleural thickening and pleural plaques but these are not considered as premalignant lesions. In rats intrapleural injection of asbestos was demonstrated to produce mesotheliomas (Wagner et al., 1969), which could be transplanted subcutaneously in syngeneic rats (Wagner et al., 1982).

Nonasbestos fibers with the same physical properties as asbestos have also been found to cause malignant mesothelioma. A high incidence of mesothelioma has been observed in an area of Turkey, where zeolite is naturally occurring in the environment (Baris et al., 1979). Potential occupational hazards were suggested for the

so called man-made mineral fibers (Wagner et al., 1986). It seems that any fiber of the appropriate diameter and length must be regarded as a potential health hazard. Although several hypotheses have been suggested (Frei, 1987), the mechanism of asbestos induced mesothelioma remains to be elucidated. One hypothesis is that asbestos causes an increased cell turnover of fibroblasts and mesothelial cells, which then become more susceptible for a carcinogenic event. Another hypothesis is that incomplete phagocytosis of asbestos fibers by macrophages causes leakage of lysosomal enzymes with a liberation of superoxide and free radicals, which are carcinogenic in experimental animals (Frei, 1987).

1.3 Clinical presentation, diagnosis and treatment

Most patients presenting with a malignant mesothelioma are men over 50 years old. Initially, these patients have nonspecific complaints like weight loss, dyspnoea or chest pain. Chest X-ray in patients with a pleural mesothelioma shows an unilateral pleural effusion and frequently pleural thickening. With progression of the malignancy, the tumor can grow through the thoracic wall, the mediastinum, the diaphragm and the lungs. Metastases may occur in lymph nodes, liver, brain and adrenal glands.

To establish the diagnosis of a patient suspected of a malignant mesothelioma, first the pleural effusion is aspirated and used for cytological investigation. When this does not lead to a definite diagnosis, histological material will be obtained by closed needle biopsy, thoracoscopy or ultimately open chest surgery.

Cytological diagnosis of malignant mesothelioma is limited by the difficulty of distinguishing by light microscopy malignant mesothelial cells from benign mesothelial cells and from adenocarcinoma cells (Whitaker et al., 1984). Additional techniques like immunocytochemistry and electron microscopy have been applied. As immunocytochemical markers antibodies against carcinoembryonic (CEA) and epithelial membrane antigen (EMA) have been proven useful (Wang et al., 1979; To et al., 1982; Whitaker et al., 1984). Malignant mesothelioma cells are seldomly positive for CEA while adenocarcinoma cells express this antigen frequently. Another marker which is positive on most adenocarcinoma cells and seems to be negative on malignant mesothelioma cells is MOC-31 (De Ley et al., 1985). EMA positive cells are found in most pleural effusions of malignant mesothelioma patients while reactive benign mesothelial cells are negative.

Ultrastructural investigations of malignant mesothelioma cells in effusions show typical mesothelial characteristics like long slender villi, glycogen granules and fat droplets in the cytoplasm. Although these features cannot establish a definite diagnosis of malignant mesothelioma, they are useful for the distinction from adenocarcinoma cells (Warhol et al., 1982).

In most cases of malignant mesothelioma histology can lead to a definite diagnosis. This, however, is dependent on the amount and quality of the material. Three different histological types of malignant mesothelioma can be distinguished:

(1) The epithelial type, consisting of cuboidal epithelial cells with a tubular or papillary growth pattern.

(2) The mesenchymal or sarcomatoid type consisting of spindle shaped fibroblastic cells which are arranged in fascicles.

(3) The mixed type exhibiting epithelial as well as mesenchymal structures.

In 50% of the malignant mesotheliomas an epithelial histology was found, in 34% a mixed type and in 16% of the cases a mesenchymal type (Hillerdal, 1983). Histochemical procedures used for identification of malignant mesothelioma are the periodic acid Schiff-diastase (PAS-D) and the hyaluronidase-alcian blue method. The PAS-D stains neutral mucopolysaccharides and is found negative in malignant mesotheliomas and in 50% of the adenocarcinomas positive. The hyaluronidase-alcian blue staining reveals hyaluronic acid in the tumor. Positivity for this staining excludes an adenocarcinoma, but only about 30% of the malignant mesotheliomas is positive. Although both methods can be helpful, in individual cases they do not always lead to a definite diagnosis (Corson et al., 1987).

Immunohistochemical staining with keratin antibodies has been considered useful in mesothelioma diagnosis but often leads to discordant results (Corson, 1987). Biochemical analysis of the cytoskeletal composition of mesothelioma cells has revealed that cytokeratin 5 could be a valuable marker for the discrimination between malignant mesothelioma and adenocarcinoma (Blobel et al., 1985). But specific antibodies against this keratin are not available yet. Coexpression of vimentin and keratin has been suggested to be useful for distinction between malignant mesothelioma and adenocarcinoma (LaRocca, 1984), but this coexpression also occurs in other cancers (Ramaekers et al., 1984). Staining with CEA and EMA as mentioned above could also be helpful to establish the diagnosis. However, even after application of all the above mentioned techniques the definite diagnosis of malignant mesothelioma is frequently made at autopsy.

Cytogenetic analysis of malignant mesothelioma cells seems to have new diagnostic prospects. Non-random chromosomal abnormalities have diagnostic and prognostic value in leukemias and malignant lymphomas (Yunis, 1983). Reports about cytogenetic analysis of malignant mesothelioma cells in pleural effusions and biopsy material are few (Mark, 1978; Gibas et al., 1986; Tiainen et al., 1988) and so far did not indicate towards a specific aberration. But standardization of the methods used and study of larger series of patients before treatment will hopefully indicate which chromosomes are primarily involved and which changes are secondary.

The median survival of malignant mesothelioma patients is approximately 12 months (Adams et al., 1986). Although several forms of therapy like surgery, radiation and chemotherapy have been applied to malignant mesotheliomas, their effect is limited (in Antman and Aisner, 1987).

1.4 References

Adams, V.I., Unni, K.K., Muhm, J.R., Rett, J.R., Ilstrup, D.M. and Bernatz, P.E. Diffuse malignant mesothelioma of pleura, diagnosis and survival in 92 cases. Cancer 58, 1540-1551, 1986.

Antman, K. and Aisner, J. Asbestos-related malignancy. Grune and Stratton, Inc., Orlando, Florida, 1987.

Baris, Y.I., Artvinli, M. and Sahin, A.A. Environmental mesothelioma in Turkey. Ann. N.Y. Acad. Sci. 330, 423-432, 1979.

Blobel, G.A., Moll, R., Franke, W.W., Kayser, K.W. and Gould, V.E. The intermediate filament cytoskeleton of malignant mesotheliomas and its diagnostic significance. Am. J. Pathol. 121, 235-247, 1985.

Corson, J.M. Pathology of malignant mesothelioma. In: Asbestos related malignancy, (Antman, K. and Aisner, J., eds.). Grune and Stratton, Inc., Orlando, Florida, 1987, pp 179-201.

Davis, J.M.G. The pathology of asbestos related disease. Thorax 39, 801-808, 1984.

Frei, E. Asbestos : a major public health challenge. In: Asbestos related malignancy (Antman, K. and Aisner, J., eds.). Grune and Stratton, Inc., Orlando, Florida, 1987, pp xvii-xx.

Gibas, Z., Li, F.P., Antman, K.H., Bernal, S., Stahel, R. and Sandberg, A.A. Chromosome changes in malignant mesothelioma. Cancer Genet. Cytogenet. 20, 191-201, 1986.

Hillerdal, G. Malignant mesothelioma 1982: Review of 4710 published cases. Br. J. Chest 77, 321-343, 1983.

LaRocca, P.J. and Rheinwald, J.G. Coexpression of simple epithelial keratins and vimentin by human mesothelium and mesothelioma in vivo and in culture. Cancer Res. 44, 2991-2999, 1984.

De Ley, L., Poppema, S., Klein Nuland, J., Ter Haar, A., Schwander, E., Ebbens, F., Postmus, P.E. and The, T.H. Neuroendocrine differentiation antigen on human lung carcinoma and Kulchitski cells. Cancer Res. 45, 2192-2200, 1985.

Lilienfeld, D.E., Mandel, J.S., Coin, P. and Schuman, C.M. Projection of asbestos related disease in the United States, 1985 - 2009. I. Cancer. Br. J. Ind. Med. 45, 283-291, 1988.

Lippmann, M. Asbestos exposure indices. Environ. Res. 46, 86-106, 1988.

Mark, J. Monosomy 14, monosomy 22 and 13q-, three chromosomal abnormalities observed in cells of two malignant mesotheliomas studied by banding technique. Acta Cytol. 22, 398-400, 1978.

McDonald, J.C. and McDonald, A.D. Epidemiology of mesothelioma from estimated incidence. Prev. Med. 6, 426-446, 1977.

Ramaekers, F., Haag, D., Jap, P. and Vooys, P.G. Immunochemical demonstration of keratin and vimentin in cytologic aspirates. Acta Cytol. 28, 385-392, 1984.

Tiainen, M., Tammilehto, L., Mattson, K. and S. Knuutila, S. Non random chromosomal abnormalities in malignant pleural mesothelioma. Cancer Genet. Cytogenet. 33, 251-274, 1988.

To, A., Dearnaley, D.P., Ormerod, M.G., Canti, G. and Coleman, D.V. Epithelial membrane antigen: its use in cytodiagnosis of malignancy in serous effusions. Am. J. Clin. Pathol. 78, 214-219, 1982.

Wagner, J.C., Sleggs, C.A. and Marchand, P. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. Brit. J. Ind. Med. 17, 260-271, 1960.

Wagner, J.C. and Berry, G. Mesotheliomas in rats following inoculation with asbestos. Br. J. Cancer 23, 567-581, 1969.

Wagner, J.C., Johnson, N.F., Brown, D.F. and Wagner, M.M.F. Histology and ultrastructure of serially transplanted rat mesotheliomas. Br. J. Cancer 46, 294-299, 1982.

Wagner, J.C. and Pooley, F.D. Mineral fibres and mesothelioma. Thorax 41, 161-166, 1986.

Wang, N.-S., Huang, S.-N. and Gold, P. Absence of carcinoembryonic antigen-like material in mesothelioma. Cancer 44, 937-943, 1979.

Warhol, M.J., Hickey, W.F. and Corson, J.M. Malignant mesothelioma: ultrastructural distinction from adenocarcinoma. Am. J. Surg. Pathol. 6, 307-314, 1982.

Whitaker, D. and Shilkin, K.B. Diagnosis of pleural malignant mesothelioma in life - a practical approach. J. Pathol. 143, 147-175, 1984.
Yunis, J.J. The chromosomal basis of human neoplasia. Science 221, 227-236, 1983.

CHAPTER 2

GROWTH FACTORS AND ONCOGENES

2.1 Growth factors

Growth regulation during embryogenesis and the maintenance of organs and tissues have to be controlled tightly. Aberrations of the normal growth control were thought to lead to the abnormal cell growth observed in tumor cells. The mechanisms that control normal growth have to be selective for different cell types and responsive to e.g. physical, hormonal and metabolic changes in the organism. Data are accumulating about the involvement of growth factors in embryonic development and normal growth regulation. Growth factors, in contrast to steroid hormones, were found to act locally and to diffuse only over a short-range. Growth factors are polypeptides that stimulate cell proliferation through binding to specific high affinity receptors (Goustin et al., 1986).

In vitro models are frequently used to study growth regulation in normal and malignant cells. Normal cells display in culture a restricted growth and an ordered growth pattern and require serum addition to the culture medium for their proliferation. These properties were thought to be related to the increased growth factor requirement of normal cells compared to their malignant counterparts, which show unlimited growth potential and disordently growth.

The purification of different growth factors has led to the development of growth factor defined culture media. Different normal cell types have been found to proliferate optimally on different combinations of growth factors. Some growth factors are highly specific for only a few cell types, e.g. the hematopoietic growth factors, while others, like the epidermal growth factor (EGF), stimulate many cell types.

Tumor cells were found to have decreased serum requirements *in vitro* (Temin, 1966) and to loose density dependent regulation compared to normal cells. These properties were found to be due to an independency of exogenous growth factors (Kaplan et al., 1982). It was proposed that tumor cells produce their own growth factors that can bind to specific receptors on the cell. This process has been called autocrine secretion compared to the endocrine and paracrine secretion models (Fig. 1). The hypothesis of autocrine secretion was supported by the first identification of a growth factor in the conditioned medium of sarcoma virus-transformed cells (Todaro and De Larco, 1978). This so-called sarcoma-derived growth factor (SGF) caused overgrowth and morphologic transformation of normal fibroblasts and stimulated anchorage independent growth. Subsequent biochemical purification of SGF showed that it consisted of two different polypeptides: transforming growth factor (TGF) α and β (Anzano et al., 1983). In recent years evidence has been found that many different tumor cells can produce growth factors. TGFα and TGFβ expression has been found for instance in colon-, epidermoid-, cervical-, renal-, prostate and mammary carcinoma cell lines (Coffey et al., 1986; Derynck et al., 1987). Expression of TGFβ only has been

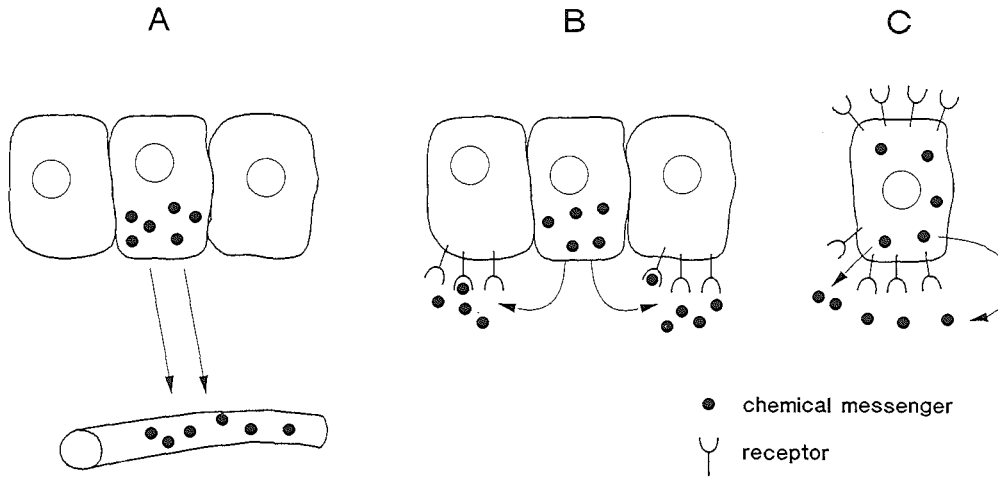

Figure 1. Schematic representation of endocrine (A), paracrine (B) and autocrine secretion (C). Adapted from Sporn and Todaro (1980).

reported for Burkitt lymphoma and T lymphoblast cell lines (Derynck et al., 1987). Insulin-like growth factors I and/or II have been found in fibrosarcomas, osteosarcomas and mammary carcinomas (Marquardt et al., 1980; Blatt et al., 1984; Huff et al., 1986).

2.2 Oncogenes

Since 1970 cancer research has been more and more focussed on the genetic damage of tumor cells. Molecular genetic studies of tumors have led to the identification of genes which are involved in malignant transformation. Investigation of acute RNA tumor viruses revealed that these viruses efficiently induced tumors in animals and were capable to transform cells in culture. In the genome of these viruses specific genes called viral oncogenes (v-*onc*) were found to be responsible for the observed oncogenicity. In 1976 it was discovered that the viral oncogenes had a strong homology with cellular sequences in the genome of vertebrate cells (Stehelin et al., 1976). These cellular oncogenes or proto-oncogenes were found to be highly conserved during evolution, suggesting an essential function. It was assumed that these oncogenes played a role in the regulation of proliferation, and after activation at the wrong time or on the wrong place could lead to malignant transformation. The first evidence supporting this hypothesis was obtained from DNA-transfection experiments

(Murray et al., 1981; Krontiris and Cooper, 1981). DNA from a human bladder cancer cell line was found to transfer tumorigenicity to NIH-3T3 mouse fibroblasts. The gene responsible for this was found to be the cellular homologue of the *ras* oncogene, which was activated by a single amino acid substitution (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982). Since then, over 40 cellular oncogenes have been identified and the list is still growing. Activation of these oncogenes can occur by proviral integration, DNA rearrangements and/or mutations. The effect of activation of a single oncogene is limited but it has been demonstrated that activation of two oncogenes in concert can transform normal fibroblasts (Land et al., 1983). Moreover, multiple cellular oncogenes were found to be activated in human tumor cell lines (Murray et al., 1981). *In vivo* experiments with transgenic mice carrying congenitally acquired oncogenes also indicate that a single oncogene is unable to induce tumors in all the cells that express it (Stewart et al., 1984). These observations support the generally accepted model of multistep carcinogenesis.

2.3 Relationship between growth factors and oncogenes

The relationship between cellular oncogenes and growth factors was established with the discovery of oncogenes that encode growth factors or growth factor receptors. The viral oncogene v-*sis* from the simian sarcoma virus (SSV) has been found to be nearly identical to the B-chain of the platelet-derived growth factor (PDGF) (Waterfield et al., 1983; Robbins et al., 1983; Deuel et al., 1983). Examples of oncogenes that encode growth factor receptors are *v-erb*B, *neu* and *fms* oncogenes. The protein product of the *v-erb*B oncogene was found to be a mutated EGF receptor (Downward et al., 1984). The *neu* oncogene, which is distantly related to *erb*B, encodes a growth factor receptor with an unknown ligand (Bargman et al., 1986), while the *fms* oncogene encodes a mutated form of the receptor for the mononuclear phagocyte growth factor CSF-1 (Sherr et al., 1985). Genetic aberrations (like mutations, amplification and translocations) can convert these proto-oncogenes to oncogenes and directly influence growth control. These observations resulted in an extension of the autocrine secretion model (Fig. 2). In this model oncogene-stimulated growth factor production can be caused by stimulation of the expression of growth factor encoding genes by oncogenes or by deregulation of a growth factor encoding oncogene (A). When the cell has the appropriate growth factor receptor a constitutive mitogenic signal will be produced so that the cell becomes independent of exogenous growth factors. Growth factor autonomy can also be obtained by receptor alteration (B) which leads to malfunctioning receptors. Finally, a change in the signal transduction from the membrane to the nucleus can lead to an aberrant signal (C). These three mechanisms can explain the observed growth factor autonomy in tumor cells.

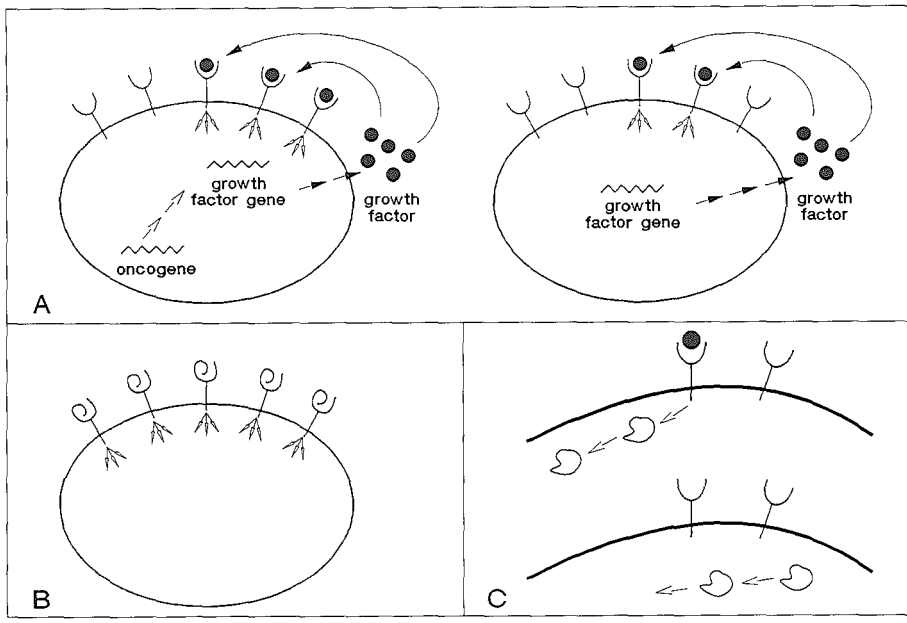

Figure 2. *Three possible mechanisms which can allow a cell to escape dependence of exogenous growth factors: by autocrine secretion (A), by receptor alteration (B) and by transducer alteration (C). Adapted from Weinberg (1985).*

2.4 Platelet-derived growth factor (PDGF)

In 1974 it was discovered that platelets release mitogens which can support growth of cells in serum-free medium which otherwise is strictly dependent on serum supplementation (Ross et al., 1974). In subsequent studies one of these mitogens was identified as platelet-derived growth factor (PDGF). Human plasma contains undetectable levels of PDGF and experiments with intravenous administration of PDGF into baboons established an half life of less than 2 minutes (Bowen-Pope et al., 1984). PDGF is thought to be locally synthesized and secreted (Ross et al., 1986).

2.4.1 Biological effects and biochemical analysis of PDGF

Several biological effects of PDGF purified from platelets have been detected in *in vitro* systems. PDGF was found to be a strong mitogen for connective tissue cells and glial cells (Ross et al., 1974; Heldin et al., 1985). In responsive cells PDGF has been found to stimulate protein and collagen synthesis and collagenase secretion

(Canalis, 1981; Owen et al., 1982; Chua et al., 1985). PDGF has also been found to be chemotactic for fibroblasts, smooth muscle cells, monocytes and neutrophils (Grotendorst et al., 1982; Seppa et al., 1982). In the latter two cell types, which do not exhibit a mitogenic response to PDGF, PDGF was found to stimulate the release of granular constituents (Tzeng et al., 1984; 1985). Vasoconstriction of arterial smooth muscle cells was observed after PDGF stimulation (Berk et al., 1986). The above mentioned biological effects of PDGF are thought to be important in embryogenesis, wound-healing and pathological conditions as arthritis and atherosclerosis (Rappolee et al., 1988; Lynch et al., 1987; Pierce et al., 1988; Rubin et al., 1988; Barrett et al., 1988).

Biochemical analysis of PDGF from human platelets revealed a molecular weight of 30 kD and a composition of two different disulphide-bound polypeptide chains, designated the A- and B-chain (Johnsson et al., 1982). The dimeric structure is essential for its function as the biological activity is lost after reduction. The PDGF B-chain has been found to be nearly identical to the transforming protein of Simian sarcoma virus (SSV) $p28^{sis}$ (Waterfield et al., 1983; Doolittle et al., 1983). This homology was an indication for the involvement of a PDGF-like growth factor in transformation and led to the study of the mechanism of SSV-transformation.

2.4.2 Simian sarcoma virus transformation, PDGF A-chain and PDGF B-chain genes

SSV has been firstly isolated from a fibrosarcoma of a woolly monkey (Theilen et al., 1971; Wolfe et al., 1971) and was found to induce sarcomas and gliomas *in vivo* (Deinhard et al., 1980) and to transform cells with PDGF receptors *in vitro*. Inhibition of the binding of PDGF to its receptor by antibodies (Johnsson et al., 1985) or suramin in SSV transformed cells reversed the transformed phenotype (Betsholtz et al., 1986a). These experiments suggest that autocrine stimulation is the basis of SSV transformation although immortalization has not been observed in SSV transformed cells (Johnsson et al., 1986).

The human cellular homologue of the viral *sis* gene, the c-*sis* or PDGF B-chain gene has been cloned (Josephs et al., 1984; Johnsson et al., 1984) and was mapped to chromosome 22q13.1 (Swan et al., 1982; Dalla Favera et al., 1982). The human PDGF A-chain gene has been cloned and mapped to chromosome 7p21-7p22 (Betsholtz et al., 1986b; Morton et al., 1987). These data are summarized in table 1. The expression of the PDGF A-chain specific mRNA of 2.8, 2.3 and 1.9 kb has been found to be independent from B-chain expression (Betsholtz et al., 1986a; Bonthron et al., 1988). The amino acid sequence homology of the PDGF A-chain with the PDGF B-chain has been found to be 60% (Johnsson et al., 1984; Josephs et al., 1984; Betsholtz et al., 1986b). In transfection experiments with PDGF A- and PDGF B-chain

TABLE 1

Chromosomal localization of human PDGF chain and PDGF receptor genes

gene	chromosomal localization	reference
PDGF A-chain	7p21-7p22	Betsholtz et al., 1986a Morton et al., 1987
PDGF B-chain (c-*sis*)	22q13.1	Swan et al., 1982 Dalla Favera et al., 1982
PDGF A-type receptor	4q11-4q12	Matsui et al., 1989
PDGF B-type receptor	5q23-5q31	Yarden et al., 1986

gene constructs phenotypical transformation and focus formation were detected in B-chain transfectants and not in A-chain transfectants (Bywater et al., 1988).

2.4.3 PDGF A-chain and B-chain expression in normal cells

Production of PDGF has been described for the following normal cell types: activated macrophages (Shimokado et al., 1985; Martinet et al., 1986), cytotrophoblasts in human placenta (Goustin et al., 1985), endothelial cells (Dicorleto and Bowen-Pope, 1983; Collins et al., 1985; 1987) and arterial smooth muscle cells (Seifert et al., 1984; Nilsson et al., 1985). In activated macrophages expression of the PDGF B-chain has been found, while about A-chain expression no data are available. The other cell types mentioned were all found to express both A- and B-chains. For PDGF in cytotrophoblasts and smooth muscle cells an autocrine function was suggested as both cell types also respond to PDGF (Heldin et al., 1988a). In injured arterial smooth muscle cells elevated PDGF production and down regulation of PDGF receptors have been described (Walker et al., 1986), which is suggestive for a role of PDGF in wound healing. Endothelial cells and macrophages do not have PDGF receptors but indications for a paracrine function are accumulating (Walker et al., 1986; Martinet et al., 1987).

2.4.4 PDGF A-chain and B-chain production in human tumor cell lines

Expression of PDGF A- and/or B-chain mRNA has been found in different types of human sarcoma cell lines, glioma cell lines, mammary carcinoma cell lines, prostate carcinoma cell lines, melanoma cell lines, non-small cell lung carcinoma cell lines and malignant mesothelioma cell lines (Table 2). In several cell lines production of PDGF was detected according to its presence in the conditioned media (Betsholtz et al.,

TABLE 2

PDGF A- and/or B-chain mRNA expression in human tumor cell lines as detected by Northern blot analysis

tumor	number of cell lines investigated	number of cell lines with A-chain expression	number of cell lines with B-chain expression	reference
sarcoma	6	ND	5	Eva et al., 1982
	8	7	3	Betsholtz et al., 1986a
glioma	5	ND	3	Eva et al., 1982
	23	23	17	Nistér et al., 1988b
mammary carcinoma	9	8	9	Peres et al., 1987
prostate carcinoma	2	2	2	Sitaras et al., 1988
malignant mesothelioma	7	6	6	Gerwin et al., 1987
melanoma	5	3	1	Westermark et al., 1986
large cell carcinoma	3	3	3	Söderdahl et al., 1988
adenocarcinoma	2	2	1	Söderdahl et al., 1988
squamous carcinoma	1	1	1	Söderdahl et al., 1988

ND, not done

1983; 1984; Nistér et al., 1984). In most cell lines expression of PDGF A-chain as well as PDGF B-chain mRNA was detected (Table 2). Whether the produced PDGF can function as an autocrine growth factor and play a role in the malignant transformation of these cell lines is dependent on the presence of PDGF receptors. In several glioma cell lines and a single osteosarcoma cell line PDGF receptors have been detected. In mammary and prostate carcinoma cell lines, which lack PDGF receptors, an autocrine function of the produced PDGF is not likely. However, a paracrine role *in vivo* has to be considered as in many tumors an increase of connective tissue growth has been observed (Seemayer et al., 1979; Kao et al., 1984). So far no data are available about the expression of PDGF receptors in the other cell lines mentioned above. Recently expression of the genes for the PDGF A-chain, the PDGF B-chain and the PDGF B-type receptor, detected by Northern blot analysis and RNA in *situ* hybridization was found in three cases of glioblastoma multiforme (Hermansson et al., 1988). So far this is the only report about expression of PDGF chain- and PDGF receptor genes in fresh solid tumor material.

TABLE 3

Identified dimeric forms of PDGF and their source

dimeric form of PDGF	source	reference
AB	human platelets	Hammacher et al., 1988
BB	porcine platelets	Strooband and Waterfield, 1984
BB	SSV transformed cells	Robbins et al., 1983
AA	human osteosarcoma cell line	Heldin et al., 1986
AA	human melanoma cell line	Westermark et al., 1986
AA	human glioma cell line	Hammacher et al., 1988

2.4.5 Functional analysis of different dimeric forms of PDGF

Although PDGF A-chain and B-chain expression has been found in many cell types it is only recently that it became possible to investigate by reversed-phase chromatography and subsequent immunoprecipitation whether the produced chains are assembled as hetero- or homodimers (Hammacher et al., 1988a). Table 3 summarizes these results and shows that all different dimeric combinations of PDGF chains have been identified. Functional analysis of different dimeric forms indicates that they have different activities. An AA homodimer, purified from the conditioned medium of a human glioma cell line, was found to have on human foreskin fibroblasts a low mitogenic activity, a low ability to induce actin reorganization and membrane ruffling and no chemotactic activity (Nister et al., 1988a), while in human fibroblasts platelet-derived AB displayed substantial activity in these respects. (Mellström et al., 1983; Nister et al., 1988a).

2.5 PDGF receptors

PDGF binds to specific cell surface receptors which are transmembrane proteins. The internal domain has protein tyrosine kinase activity (Ek et al., 1982; Yarden et al., 1986), while the external domain is involved in ligand binding. Ligand binding induces activation of the tyrosine kinase domain. Although several cellular effects of PDGF stimulation have been reported, like increase of cytoplasmic free Ca^{2+} concentration (Moolenaar et al., 1984) and stimulation of protein kinase C (Rozengurt et al., 1983), the exact mechanism of signal transduction is still unknown.

With the availability of recombinant dimeric AA and BB it has become possible to investigate the specificity of PDGF receptors for these different dimeric forms.

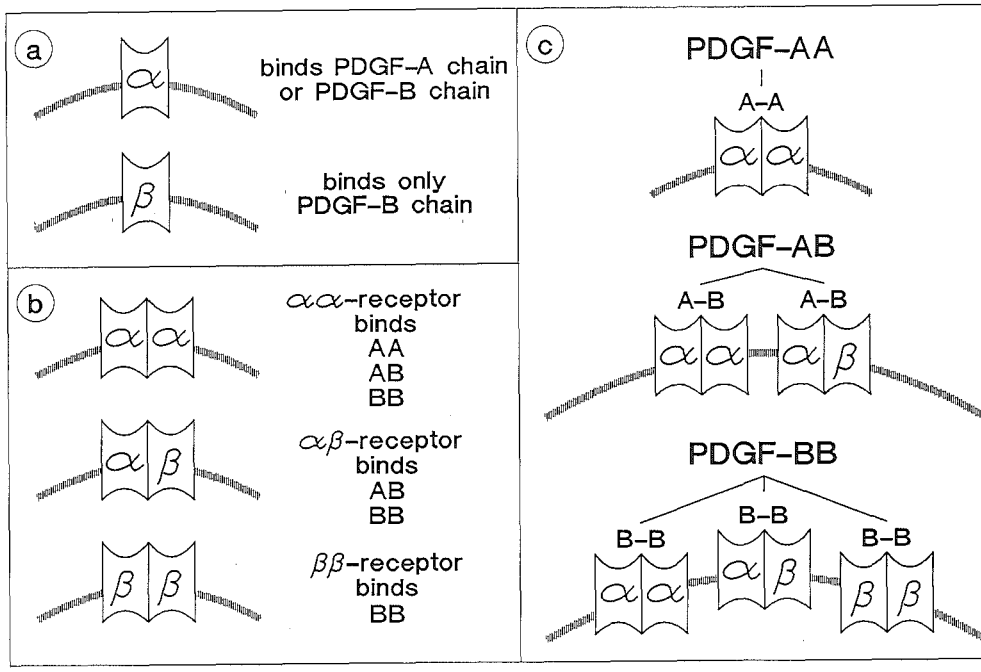

Figure 3. *The PDGF receptor subunit model. a. The two types of PDGF receptor subunits that are found: α-subunits that can bind PDGF A- and B-chains and β-subunits that bind PDGF-B chains. b. Receptor types that can be composed of the subunits: αα-receptors, αβ-receptors and ββ-receptors. c. Receptor binding: PDGF-AA can only bind to PDGF αα-receptors, PDGF-AB can bind to PDGF αα- or αβ-receptors and PDGF-BB can bind to all three receptors. Adapted from Seifert et al. (1989).*

Binding experiments to cultured cells have revealed the already suggested (Nistér et al., 1988a; Hart et al., 1988) existence of two different PDGF receptor types, the PDGF A-type and the PDGF B-type receptor (Heldin et al., 1988b; Hart et al., 1988). The A-type receptor was found to bind AA, AB and BB, whereas the B-type receptor binds BB with high affinity, AB with lower affinity and AA not at all (Heldin et al., 1988b).

A human PDGF receptor has been recently cloned (Gronwald et al., 1988; Claesson-Welsh et al., 1988). After expression in CHO cells this receptor was found to be the B-type receptor with a molecular weight of 170-185 kD, which was synthesized after glycosylation from a 160 kD molecule (Hart et al., 1987; Keating and Williams, 1987; Claesson-Welsh et al., 1988). In recent experiments PDGF was found

to induce dimerization of two PDGF receptors and subsequent activation of the tyrosine kinase domain of the dimerized receptor (Heldin et al., 1989; Seifert et al., 1989). According to these observations the so called 'PDGF receptor subunit model' was proposed (Seifert et al., 1989). In this model the PDGF A- and B-type receptors are called subunit α and β, respectively. These subunits dimerize to form three distinct PDGF receptors that have different specificities for binding the three isoforms of PDGF (Fig. 3). The role of the detected dimerization in signal transduction remains to be elucidated. The PDGF A-type receptor has been recently cloned (Matsui et al., 1989; Claesson-Welsh et al., 1989b), and characterized by immunoprecipitation. It was found to be a molecule of 170 kD after glycosylation of a 140 kD molecule (Claesson-Welsh et al., 1989a). The mitogenic response after binding of PDGF-AA to the A-type receptor in human fibroblasts was found to be low compared to binding of PDGF-BB to the B-type receptor (Heldin et al., 1988b). Therefore, the A-type receptor has possibly another function.

The PDGF A- and B-type receptors have both an extracellular part of five immunoglobulin-like domains, a transmembrane domain and an intracellular protein tyrosine kinase domain. This protein tyrosine kinase domain is divided into two parts, TK1 and TK2, by an intervening sequence. The amino acid sequence identity between the two PDGF receptors is most conserved in the tyrosine kinase domains (85% and 75%), while between the extracellular ligand binding domains of the two receptors only 31% homology has been found. The PDGF A- and B-type receptor genes are localized on the chromosomes 4 and 5, respectively (table 1).

2.6 References

Anzano, M.A., Roberts, A.B., Smith, J.M., Sporn, M.B. and De Larco, J.E. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and type β transforming growth factors. Proc. Natl. Acad. Sci. USA 80, 6264-6268, 1983.

Bargmann, C.I., Hung, M.-C. and Weinberg, R.A. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature 319, 226-230, 1986.

Barnes, D. and Sato, G. Serum-free cell culture: a unifying approach. Cell 22, 649-655, 1980.

Barrett, T.B. and Benditt, E.P. Platelet-derived growth factor gene expression in human atherosclerotic plaques and normal artery wall. Proc. Natl. Acad. Sci. USA 85, 2810-2814, 1988.

Berk, B.C., Alexander, R.W., Brock, T.A., Gimbrone, M.A. and Webb, R.C. Vasoconstriction: a new activity for platelet-derived growth factor. Science 232, 87-90, 1986.

Betsholtz, C., Heldin, C.-H., Nistér, M., Ek, B., Wästeson, A. and Westermark, B. Synthesis of a PDGF-like growth factor in human glioma and sarcoma cells suggests the expression of the cellular homologue to the transforming protein of Simian sarcoma virus. Biochem. Biophys. Res. Commun. 117, 176-182, 1983.

Betsholtz, C., Westermark, B., Ek, B. and Heldin, C.-H. Coexpression of a PDGF-like growth factor and PDGF receptors in a human osteosarcoma cell line: implications for autocrine receptor activation. Cell 39, 447-457, 1984.

Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L., Knott, T.J. and Scott, J. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. Nature 320, 695-699, 1986a.

Betsholtz, C., Johnsson, A., Heldin, C.-H. and Westermark, B. Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. Proc. Natl. Acad. Sci. USA 83, 6440-6444, 1986b.

Blatt, J., White, C., Dienes, S., Friedman, H. and Foley, T.P. Production of an insulin-like growth factor by osteosarcoma. Biochem. Biophys. Res. Comm. 123, 373-376, 1984.

Bonthron, D.T., Morton, C.C., Orkin, S.H. and Collins, T. Platelet-derived growth factor A chain: Gene structure, chromosomal location, and basis for alternative mRNA splicing. Proc. Natl. Acad. Sci. USA 85, 1492-1496, 1988.

Bowen-Pope, D.F., Malpass, T.W., Foster, D.M. and Ross, R. Platelet-derived growth factor *in vivo*: levels, activity and rate of clearance. Blood 64, 458-469, 1984.

Bronzert, D.A., Pantazis, P., Antoniades, H.N., Kasid, A., Davidson, N., Dickson, R.B. and Lippman, M.E. Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. Proc. Natl. Acad. Sci. USA 84, 5763-5767, 1987.

Bywater, M., Rorsman, F., Bongcam-Rudloff, E., Mark, G., Hammacher, A., Heldin, C.-H., Westermark, B. and Betsholtz, C. Expression of recombinant platelet-derived growth factor A- and B-chain homodimers in rat-1 cells and human fibroblasts reveals differences in protein processing and autocrine effects. Mol. Cell. Biol. 8, 2753-2762, 1988.

Canalis, E. Effect of platelet-derived growth factor on DNA and protein synthesis in cultured rat calvaria. Metabolism 30, 970-975, 1981.

Chua, C.C., Geiman, D.E., Keller, G.H. and Ladda, R.L. Induction of collagenase secretion in human fibroblast cultures by growth promoting factors. J. Biol. Chem. 260, 5213-5216, 1985.

Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C. and Heldin, C.-H. cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. Mol. Cell. Biol. 8, 3476-3486, 1988.

Claesson-Welsh, L., Hammacher, A., Westermark, B., Heldin, C.-H. and Nistér, M. Identification and structural analysis of the A-type receptor for platelet-derived growth factor, similarities with the B-type receptor. J. Biol. Chem. 264, 1742-1747, 1989a.

Claesson-Welsh, L., Eriksson, A., Westermark, B. and Heldin, C.-H. cDNA cloning and expression of the human A-type PDGF receptor establishes structural similarity to the B-type receptor. Proc. Natl. Acad. Sci. USA, in press, 1989b.

Coffey Jr., R.J., Shipley, G.D. and Moses, H.L. Production of transforming growth factors by human colon cancer lines. Cancer Res. 46, 1164-1169, 1986.

Collins, T., Ginsburg, D., Boss, J.M., Orkin, S.H. and Pober, J.S. Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis. Nature 316, 748-750, 1985.

Collins, T., Pober, J.S., Gimbrone, Jr. M.A. Hammacher, A., Betsholtz, C., Westermark, B. and Heldin, C.-H. Cultured human endothelial cells express platelet-derived growth factor A chain. Am. J. Path. 126, 7-12, 1987.

Dalla-Favera, R., Gallo, R.C., Giallongo, A. and Croce, C.M. Chromosomal localization of the human homolog (c-sis) *of the simian sarcoma virus onc* gene. Science 218, 686-688, 1982.

Deinhardt, F. Biology of primate retroviruses. In: Klein, Viral oncology. Raven Press, New York, 1980, pp 357-398.

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S. and Berger, W.H. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res. 47, 707-712, 1987.

Deuel, T.F., Huang, J.S., Huang, S.S., Stroobant, P. and Waterfield, M.D. Expression of a platelet-derived growth factor-like protein in simian sarcoma virus transformed cells. Science 221, 1348-1350, 1983.

Dicorleto, P.E. and Bowen-Pope, D.F. Cultured endothelial cells produce a platelet-derived growth factor-like protein. Proc. Natl. Acad. Sci. USA 80, 1919-1923, 1983.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. Simian sarcoma virus *onc* gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221, 275-277, 1983.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. Close similarity of epidermal growth factor receptor and *v-erb*B oncogene protein sequences. Nature 307, 521-527, 1984.

Ek, B., Westermark, B., Wästeson, A. and Heldin, C.-H. Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor. Nature 295, 419-420, 1982.

Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C. and Aaronson, S.A. Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells. Nature 295, 116-119, 1982.

Gerwin, B.I., Lechner, J.F., Reddel, R.R., Roberts, A.B., Robbins, K.C., Gabrielson, E.W. and Harris, C.C. Comparison of production of transforming growth factor-β and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47, 6180-6184, 1987.

Goustin, A.S., Betsholtz, C., Pfeifer-Ohlsson, S., Persson, H., Rydnert, J., Bywater, M., Holmgren, G., Heldin C.-H., Westermark, B. and Ohlsson, R. Coexpression of the *sis and myc* proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth. Cell 41, 301-312, 1985.

Goustin, A.S., Leof, E.B., Shipley, G.D. and Moses, H.L. Growth factors and cancer. Cancer Res. 46, 1015-1029, 1986.

Gronwald, R.G.K., Grant, F.J., Haldeman, B.A., Hart, C.E., O'Hara, P.J., Hagen, F.S., Ross, R., Bowen-Pope, D.F. and Murray, M.J. Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: Evidence for more than one receptor class. Proc. Natl. Acad. Sci. USA 85, 3435-3439, 1988.

Grotendorst, G.R., Chang, T., Seppä, H.E.J., Kleinman, H.K. and Martin, G.R. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J. Cell. Physiol. 113, 261-266, 1982.

Hammacher, A., Nistér, M., Westermark, B. and Heldin, C.-H. A human glioma cell line secretes three structurally and functionally different dimeric forms of platelet-derived growth factor. Eur. J. Biochem. 176, 179-186, 1988a.

Hammacher, A., Hellman, U., Johnsson, A., Östman, A., Gunnarsson, K., Westermark, B., Wästeson, A. and Heldin, C.-H. A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. J. Biol. Chem. 263, 16493-16498, 1988b.

Hart, C.E., Seifert, R.A., Ross, R. and Bowen-Pope, D.F. Synthesis, phosphorylation, and degradation of multiple forms of the platelet-derived growth factor receptor studied using a monoclonal antibody. J. Biol. Chem. 262, 10780-10785, 1987.

Hart, C.E., Forstrom, J.W., Kelly, J.D., Seifert, R.A., Smith R.A., Ross, R., Murray, M.J. and Bowen-Pope, D.F. Two classes of PDGF receptor recognize different isoforms of PDGF. Science 240, 1529-1531, 1988.

Heldin, C.-H., Wästeson, A. and Westermark, B. Platelet-derived growth factor. Mol. Cell. Endocr. 39, 169-187, 1985.

Heldin, C.-H., Johnsson, A., Wennergren, S., Wernstedt, C., Betsholtz, C. and Westermark, B. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Nature 319, 511-514, 1986.

Heldin, C.-H., Hammacher, A., Nistér, M. and Westermark, B. Structural and functional aspects of platelet-derived growth factor. Br. J. Cancer 57, 591-593, 1988a.

Heldin, C.-H., Bäckström, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nister, M. and Westermark, B. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J 7, 1387-1393, 1988b.

Hermansson, M., Nistér, M., Betsholtz, C., Heldin, C.-H., Westermark, B. and Funa, K. Endothelial cell hyperplasia in human glioblastoma: Coexpression of mRNA for platelet-derived growth factor (PDGF) B chain and PDGF receptor suggests autocrine growth stimulation. Proc. Natl. Acad. Sci. USA 85, 7748-7752, 1988.

Huff, K.K., Kaufman, D., Gabbay, K.H., Spencer, E.M., Lippman, M.E. and Dickson, R.B. Secretion of an insulin-like growth factor I related protein by human breast cancer cells. Cancer Res. 46, 4613-4619, 1986.

Johnsson, A., Heldin, C.-H., Westermark, B. and Wästeson, A. Platelet-derived growth factor: identification of constituent polypeptide chains. Biochem. Biophys. Res. Commun. 104, 66-74, 1982.

Johnsson, A., Heldin, C.-H., Wästeson, A., Westermark, B., Deuel, T.F., Huang, J.S., Seeburg, P.H., Gray, A., Ullrich, A., Scrace, G., Stroobant, P. and Waterfield, M.D. The c-*sis* gene encodes a precursor of the B chain of platelet-derived growth factor. EMBO J. 3, 921-928, 1984.

Johnsson, A., Betsholtz, C., Von der Helm, K., Heldin, C.-H. and Westermark B. Platelet-derived growth factor agonist activity of a secreted form of the v-*sis* oncogene product. Proc. Natl. Acad. Sci. USA 82, 1721-1725, 1985.

Johnsson, A., Betsholtz, C., Heldin, C.-H. and Westermark, B. The phenotypic characteristics of simian sarcoma virus-transformed human fibroblasts suggest that the v-*sis* gene product acts solely as a PDGF receptor agonist in cell transformation. EMBO J. 5, 1535-

1541, 1986.
Josephs, S.F., Guo, C., Ratner, L. and Wong-Staal, F. Human proto-oncogene nucleotide sequences corresponding to the transforming region of Simian sarcoma virus. Science 223, 487-490, 1984.
Kao, R.T., Hall, J., Engel, L. and Stern, R. The matrix of human breast tumor cells is mitogenic for fibroblasts. Am. J. Path. 115, 109-116, 1984.
Kaplan, P.L., Anderson, M. and Ozanne, B. Transforming growth factors production enables cells to grow in the absence of serum: an autocrine system. Proc. Natl. Acad. Sci. USA 79, 485-489, 1982.
Keating, M.T. and Williams, L.T. Processing of the platelet-derived growth factor receptor. J. Biol. Chem. 262, 7932-7937, 1987.
Kohler, N. and Lipton, A. Platelets as a source of fibroblast growth-promoting activity. Exp. Cell. Res. 87, 297-301, 1974.
Krontiris, T.G. and Cooper, G.M. Transforming activity of human tumor DNAs. Proc. Natl. Acad. Sci. USA 78, 1181-1184, 1981.
Land, H., Parada, L.F. and Weinberg, R.A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 306, 596-602, 1983.
Lynch, S.E., Nixon, J.C., Colvin, R.B. and Antoniades, H.N. Role of platelet-derived growth factor in wound healing: Synergistic effects with other growth factors. Proc. Natl. Acad. Sci. USA 84, 7696-7700, 1987.
Martinet, Y., Bitterman, P.B., Mornex, J.-F., Grotendorst, G.R., Martin, G.R. and Crystal, R.G. Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity. Nature 319, 158-160, 1986.
Martinet, Y., Rom, W.N., Grotendorst, G.R., Martin, G.R. and Crystal, R.G. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. New Engl. J. Med. 317, 202-209, 1987.
Marquardt, H., Wilson, G.L. and Todaro, G.J. Isolation and characterization of a multiplication stimulating (MSA) like polypeptide produced by a human fibrosarcoma cell line. J. Biol. Chem. 255, 9177-9181, 1980.
Matsui, T., Heidaran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J. and Aaronson, S. Isolation of a novel receptor cDNA established the existence of two PDGF receptor genes. Science 243, 800-803, 1989.
Mellström, K., Höglund, A.-S., Nistér, M., Heldin, C.-H., Westermark, B. and Lindberg, U. The effect of platelet-derived growth factor on morphology and motility of human glial cells. J. Muscle Res. Cell Motil. 4, 589-609, 1983.
Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. Growth factors immediately raise cytoplasmic free Ca^{2+} in human fibroblasts. J. Biol. Chem. 259, 8066-8069, 1984.
Morton, C.C., Bonthron, D.T., Collins, T. Platelet-derived growth factor A chain is assigned to 7p22-p21. Human Gene Mapping 9, 664, 1987.
Murray, M.J., Shilo, B.-Z., Shih, C., Cowing, D., Hsu, H.W. and Weinberg, R.A. Three different human tumor cell lines contain different oncogenes. Cell 25, 355-361, 1981.
Nilsson, J., Sjölund, M., Palmberg, L., Thyberg, J. and Heldin, C.-H. Arterial smooth muscle cells in primary culture produce a platelet-derived growth factor-like protein. Proc. Natl. Acad. Sci. USA 82, 4418-4422, 1985.
Nistér, M., Heldin, C.-H., Wasteson, A. and Westermark, B. A glioma-derived analog to platelet-derived growth factor: Demonstration of receptor competing activity and immunological crossreactivity. Proc. Natl. Acad. Sci. USA 81, 926-930, 1984.
Nistér, M., Hammacher, A., Mellström, K., Siegbahn, A., Rönnstrand, L., Westermark, B. and Heldin, C.-H. A glioma-derived PDGF A chain homodimer has different functional activities from a PDGF AB heterodimer purified from human platelets. Cell 52, 791-799, 1988a.
Nistér, M., Libermann, T.A., Betsholtz, C., Pettersson, M., Claesson-Welsh, L., Heldin, C.-H., Schlessinger, J. and Westermark, B. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-α and their receptors in human malignant glioma cell lines. Cancer Res. 48, 3910-3918, 1988b.
Owen, A.J., Geyer, R.P. and Antoniades, H.N. Human platelet-derived growth factor stimulates amino acid transport and protein synthesis by human diploid fibroblasts in plasma-free media. Proc. Natl. Acad. Sci. USA 79, 3203-3207, 1982.
Peres, R., Betsholtz, C., Westermark, B. and Heldin, C.-H. Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. Cancer Res. 47, 3425-3429, 1987.
Pierce, G.F., Mustoe, T.A., Senior, R.M., Reed, J., Griffin, G.L., Thomason, A. and Deuel, T.F. *In vivo* incisional wound healing augmented by platelet-derived growth factor and recombinant c-*sis* gene homodimeric proteins. J. Exp. Med. 167, 974-987, 1988.

Rappolee, D.A., Brenner, C.A., Schultz, R., Mark, D. and Werb, Z. Developmental expression of PDGF, TGF-α, and TGF-β genes in preimplantation mouse embryos. Science 241, 1823-1825, 1988.
Reddy, E.P., Reynolds, R.K., Santos, E. and Barbacid, M. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 300, 149-152, 1982.
Robbins, K.C., Antoniades, H.N., Devare, S.G., Hunkapiller, M.W. and Aaronson, S.A. Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. Nature 305, 605-608, 1983.
Ross, R., Glomset, J., Kariya, B. and Harker, L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells *in vitro*. Proc. Natl. Acad. Sci USA 71, 1207-1210, 1974.
Ross, R., Raines, E.W. and Bowen-Pope, D.F. The biology of platelet-derived growth factor. Cell 46, 155-169, 1986.
Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A. Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a M_r 80,000 protein in intact quiescent 3T3 cells. Proc. Natl. Acad. Sci. USA 80, 7244-7248, 1983.
Rubin, K., Terracio, L., Rönnstrand, L., Heldin, C.-H. and Klareskog, L. Expression of platelet-derived growth factor receptors is induced on connective tissue cells during chronic synovial inflammation. Scand. J. Immunol. 27, 285-294, 1988.
Seemayer, T.A., Lagace, R., Schurch, W. and Tremblay, G. Myofibroblasts in the stroma of invasive and metastatic carcinoma. Am. J. Surg. Pathol. 3, 525-533, 1979.
Seifert, R.A., Schwartz, S.M. and Bowen-Pope, D.F. Developmentally regulated production of platelet-derived growth factor-like molecules. Nature 311, 669-671, 1984.
Seifert, R.A., Hart, C.E., Phillips, P.E., Forstrom, J.W., Ross, R., Murray, M.J. and Bowen-Pope, D. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. J. Biol. Chem. 264, 8771-8778, 1989.
Seppä, H., Grotendorst, G., Seppä, S., Schiffmann, E. and Martin, G.R. Platelet-derived growth factor is chemotactic for fibroblasts. J. Cell. Biol. 92, 584-588, 1982.
Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T. and Stanley, E.R. The *c-fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41, 665-676, 1985.
Shimokado, K., Raines, E.W., Madtes, D.K., Barrett, T.B., Benditt, E.P. and Ross, R. A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell 43, 277-286, 1985.
Sitaras, N.M., Sariban, E., Bravo, M., Pantazis, P. and Antoniades, H.N. Constitutive production of platelet-derived growth factor-like proteins by human prostate carcinoma cell lines. Cancer Res. 48, 1930-1935, 1988.
Söderdahl, G., Betsholtz, C., Johansson, A., Nilsson, K. and Bergh, J. Differential expression of platelet-derived growth factor and transforming growth factor genes in small- and non-small-cell human lung carcinoma lines. Int. J. Cancer 41, 636-641, 1988.
Sporn, M.B. and Todaro, G.J. Autocrine secretion and malignant transformation of cells. New Engl. J. Med. 303, 878-880, 1980.
Stehelin, D., Varmus, H.E., Bishop, J.M. and Vogt, P.K. DNA related to the transforming genes of avian sarcoma viruses is present in normal avian DNA. Nature 260, 170-173, 1976.
Stewart, T.A., Pattengale, P.K. and Leder, P. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 38, 627-637, 1984.
Stroobant, P. and Waterfield, M.D. Purification and properties of porcine platelet-derived growth factor. EMBO J. 12, 2963-2967, 1984.
Swan, D.C., McBride, O.W., Robbins, K.C., Keithley, D.A., Reddy, E.P. and Aaronson, S.A. Chromosomal mapping of the simian sarcoma virus *onc* gene analogue in human cells. Proc. Natl. Acad. Sci. USA 79, 4691-4695, 1982.
Tabin, C.J., Bradley, S.M., Bargmann, C.I., Weinberg, R.A., Papageorge, A.G., Scolnick, E.M., Dhar, R., Lowy, D.R. and Chang, E.H. Mechanism of activation of a human oncogene. Nature 300, 143-149, 1982.
Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M. and Wigler, M. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature 300, 762-765, 1982.
Temin, H.M. Studies on carcinogenesis by avian sarcoma viruses. III. The differential effect of serum and polyanions on multiplication of uninfected and converted cells. J. Natl. Cancer Inst. 37, 167-175, 1966.
Theilen, G.H., Gould, D., Fowler, M. and Dungworth, D.L. C-type virus in tumor tissue of

a woolly monkey (Lagothrix spp.) with fibrosarcoma. J. Natl. Cancer Inst. 47, 881-889, 1971.

Todaro, G.J. and De Larco, J.E. Growth factors produced by Sarcoma virus-transformed cells. Cancer Res. 38, 4147-4154, 1978.

Tzeng, D.Y., Deuel, T.F., Huang, J.S., Senior, R.M., Boxer, L.A. and Baehner, R.L. Platelet-derived growth factor promotes polymorphonuclear leukocyte activation. Blood 64, 1123-1128, 1984.

Tzeng, D.Y., Deuel, T.F., Huang, J.S. and Baehner, R.L. Platelet-derived growth factor promotes human peripheral monocyte activation. Blood 66, 179-183, 1985.

Walker, L.N., Bowen-Pope, D.F., Ross, R. and Reidy, M.A. Production of platelet-derived growth factor-like molecules by cultured arterial smooth muscle cells accompanies proliferation after arterial injury. Proc. Natl. Acad. Sci. USA 83, 7311-7315, 1986.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J.S. and Deuel, T.F. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304, 35-39, 1983.

Westermark, B., Johnsson, A., Paulsson, Y., Betsholtz, C., Heldin, C.-H., Herlyn, M., Rodeck, U. and Koprowsky, H. Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor. Proc. Natl. Acad. Sci. USA 83, 7197-7200, 1986.

Westermark, B., Johnsson, A., Betsholtz, C. and Heldin, C.-H. Biological properties of simian sarcoma virus and its oncogene product. Contr. Oncol. 24, 51-61, 1987.

Weinberg, R.A. The action of oncogenes in the cytoplasm and nucleus. Science 230, 770-776, 1985.

Wolfe, L.G., Deinhardt, F., Theilen, G.H., Rabin, H., Kawakami, T. and Bustad, L.K. Induction of tumors in marmoset monkeys by simian sarcoma virus, Type 1 (Lagothrix): A preliminary report. J. Natl. Cancer Inst. 47, 1115-1120, 1971.

Yarden, Y., Escobedo, J.A., Kuang, W.-J., Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E., Harkins, R.N, Francke, U., Fried, V.A., Ullrich, A. and Williams, L.T. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature 323, 226-232, 1986.

CHAPTER 3

INTRODUCTION TO THE EXPERIMENTAL WORK

In this thesis the clinical, diagnostic and cell biological aspects of human malignant mesothelioma are studied. The purpose of this study was to improve the diagnosis of human malignant mesothelioma on the one hand and to investigate the growth regulation and transformation of normal and malignant mesothelial cells on the other hand.

In order to investigate whether the epidemiology of the malignant mesothelioma patients in the areas of Rotterdam and Vlissingen is similar to other studied groups medical records of 124 malignant mesothelioma patients were studied. Chapter 4 describes this analysis with respect to asbestos exposure, duration of exposure, survival, radiologic findings and histopathology.

Diagnosis of malignant mesothelioma on effusions by routine cytology is hampered by the lack of specific morphological characteristics of malignant mesothelioma cells. Improvement of diagnosis was approached by the search for specific tumor markers. Initially we tried to produce monoclonal antibodies, which specifically recognize malignant mesothelioma cells. These attempts, however, were unsuccessful. Subsequently, we investigated whether epithelial membrane antigen (EMA) was useful for the identification of malignant mesothelioma cells. However, antibodies against EMA recognized malignant epithelial and mesothelial cells. Then we investigated whether the combination of EMA expression and ultrastructural features was of diagnostic significance for malignant mesothelioma (chaper 5.1).

Secondly, we studied the cytogenetics of malignant mesothelioma in order to find out whether these cells have specific chromosomal aberrations. Cytogenetic analysis was performed on patient material of 40 malignant mesotheliomas. Furthermore, the usefulness of cytogenetics for the distinction between effusions of metastatic carcinoma, lung adenocarcinoma and non-malignant effusions was evaluated.

Cell biological investigations of malignant mesothelioma could lead to more insight into the properties and growth regulation of malignant mesothelioma cells and ultimately to new diagnostic methods.

As viable tumor tissues of malignant mesothelioma for cell biological studies are difficult to obtain a panel of *in vitro* growing human malignant mesothelioma cell lines was established. In chapter 6.1 the method used for the establishment of seventeen malignant mesothelioma cell lines has been described since such a large panel of malignant mesothelioma cell lines has not been reported so far. Three of these malignant mesothelioma cell lines were characterized with regard to their growth characteristics, cytogenetic aberrations and cytokeratin expression.

In chapter 7.1 we show by Northern blot analysis that the PDGF A-chain and B-chain genes are expressed in ten malignant mesothelioma cell lines. To investigate

whether expression of the c-*sis* oncogene is detectable in freshly obtained tumor material Northern blot analysis of patient material of eight malignant mesotheliomas was performed (chapter 7.2). If the by malignant mesothelioma cell lines produced PDGF functions as an autocrine growth factor in these cell lines expression of the appropriate receptors is a prerequisite. In chapter 7.3 the expression of PDGF A- and B-type receptors in malignant mesothelioma cell lines was studied by Northern blot analysis and binding experiments. Furthermore, the presence of the PDGF B-type receptor was investigated by immunoprecipitation and immunoelectron microscopy.

In chapter 8 the results of the experimental work are discussed in the context of the literature.

CHAPTER 4

MALIGNANT PLEURAL MESOTHELIOMA IN THE SOUTHWESTERN PART OF THE NETHERLANDS

Teun van Gelder[a], Henk C. Hoogsteden[b], Marjan A. Versnel[c], Ed J. van Hezik[d], Jan P. Vandenbroucke[e], Hans T. Planteydt[f].

Department of Internal Medicine[a], Department of Pulmonary Medicine[b], Department of Immunology[c] of the Academic Hospital Dijkzigt and Erasmus University in Rotterdam, Department of Pulmonary Medicine, Regional Hospital Vlissingen[d], Department of Clinical Epidemiology[e] of the University of Leiden and Department of Pathology[f] of the Streeklaboratorium Zeeland, Middelburg, The Netherlands.

European Respiratory Journal, in press.

SUMMARY

This report is the result of an analysis of the medical records of 124 patients presenting with a malignant pleural mesothelioma. Information about asbestos exposure was available in 104 of them, which appeared to be positive in 95 (91%). The median duration of exposure was 33.6 years. The median latent period was 41.2 years. The median survival was 11 months while different ways of treatment could not prolong survival. The most common radiologic findings were pleural effusions, while in some patients contralateral effusions or pleural thickening was found. Pleural plaques or asbestosis were seen in a minority of the patients. In this series a relatively high percentage of mixed type mesotheliomas was found (56%). Large biopsies often showed both epithelial and connective tissue type elements. Concerning diagnostic procedures we recommend to start with cytology of pleural fluid, which can easily be obtained together with an Abrams biopsy. If this does not give a definite diagnosis, thoracoscopy or thoracotomy is indicated.

INTRODUCTION

Many studies have confirmed the relationship between asbestos exposure and the occurrence of malignant mesothelioma (1,2). It is now clear that exposure to asbestos may lead to a number of pathologic conditions, including benign abnormalities (3,4) and involvement of other sites than lung or pleura. A long latency period of up to 60 years between the first exposure to asbestos and the development of a malignant mesothelioma has been described. Incidence rates for pleural mesothelioma among men are increasing (5-7). The substantial increase of the frequency of pleural mesothelioma among males versus the stable trend for females argues against any large impact of diagnostic or coding changes (8).

Many shipyard workers have been exposed to asbestos in the past (mainly crocidolite) with a consequently increased frequency of malignant mesothelioma. In many coastal regions incidences of mesothelioma are found that belong to the worlds highest (9). This is also true for The Netherlands where two studies already reported an increased incidence in the Southwestern part of The Netherlands (10,11). The here reported study is an analysis of 124 pleural mesotheliomas in the areas of Vlissingen and Rotterdam, in the recent past both known for their large shipbuilding industries.

METHODS

This study is an analysis of 124 patients presenting with a malignant pleural mesothelioma. The patients were seen in two centers. Seventy-one were seen in the University Hospital in Rotterdam, while the other patients (53) were diagnosed in the Regional Hospital in Vlissingen. These 124 patients probably form about 80 to 90% of all patients that presented with a malignant mesothelioma in the Southwestern part of the Netherlands between 1962 and 1985.

For the histopathological diagnosis of mesothelioma the recommendations of the Commission of the European Communities were applied (12). Reevaluation of all histologic specimens was done by the Dutch Mesothelioma Panel. For the certainty of the diagnosis the following categories were used:

A. Definite malignant mesothelioma - no doubt as to the histopathological diagnosis.
B. Probable malignant mesothelioma - the reason for the hesitation may be lack of material, bad quality, lack of differentiation, absence of certain histological details which give rise to slight doubt.
C. Possible malignant mesothelioma - the diagnosis cannot be denied but there is insufficient evidence to come to a positive conclusion.
D. Improbable malignant mesothelioma - probably not a mesothelioma but the diagnosis cannot be absolutely denied.
E. Definitely not a malignant mesothelioma.

In categories D and E an other diagnosis should be suggested or made. Only the histopathologic categories A and B were included in the study. The analysis includes clinical, diagnostic and histologic aspects.

RESULTS

The study group consisted of 120 men (97%) and 4 women (3%). Sixty-nine (56%) mesotheliomas arose on the right side of the thorax, 51 (41%) on the left side, while three (2%) mesotheliomas were double sided. One patient had a simultaneous primary pleural- and peritoneal mesothelioma. The median age of the patients at the time of diagnosis was 64.7 yrs. (range 26-86 yrs). Almost eighty percent (87/112 = 78%) had ages between 50 and 75 years.

No occupational information was available in twenty patients. Of the other 104 many had been working in a shipyard for at least several years (62/104 = 60%). In 95 patients (91%) a positive history of occupational asbestos exposure was found. The

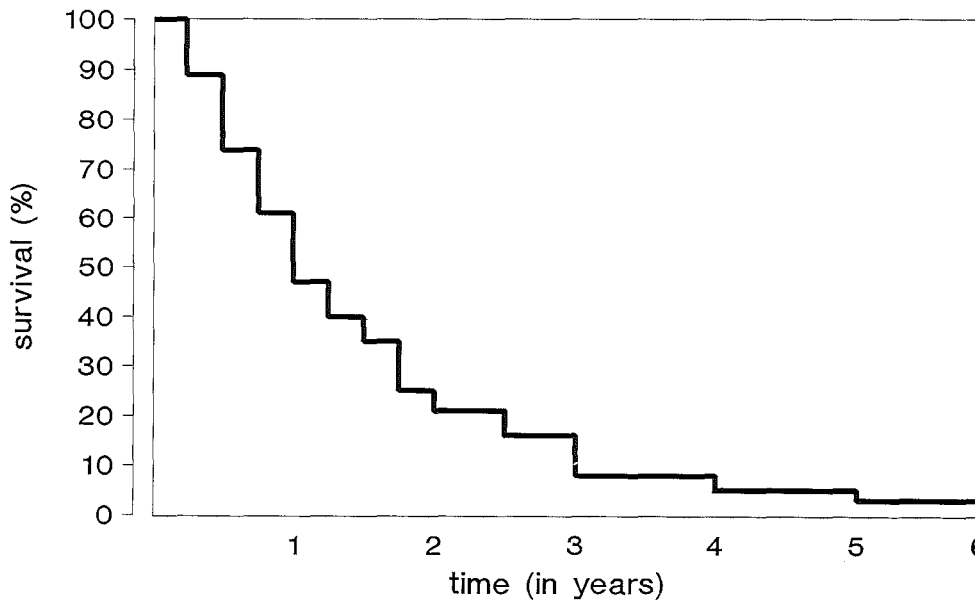

Figure 1. Survival in time of 124 patients with malignant mesothelioma.

median duration of asbestos exposure was 33.6 yrs. (range 1-52 yrs.). The latent period, defined as the interval between the beginning of exposure and the occurrence of the first symptoms, also showed a wide range. The median latent period was 41.2 yrs. (range 17- 68 yrs.). The histopathologic diagnosis was known in all subjects. The mixed type was the most prevalent and found in 68 of 124 patients (55%). In 40 (32%) patients the mesothelioma was of the epithelial type, while in 16 (13%) it was of the connective tissue type. In 102 cases the diagnosis was entitled as "definite", whereas the other 22 were "probable".

The most frequently presenting symptoms were dyspnea (81%), chest pain (73%) and weight loss (66%). Cough was found in 53% of the patients. Six times (5%) a vena cava superior syndrome was one of the presenting symptoms. In 5 patients (4%) there were palpable lymph nodes on presentation. Swallowing complaints were found in another 4 patients (3%).

The radiological findings are reported in table 1. Ipsilateral pleural effusion and pleural thickening were found most frequently.

TABLE I

Radiographic findings in 124 patients with a malignant pleural mesothelioma on presentation.

		number	%
Pleural effusion	ipsilateral	109	88
	contralateral	6	5
Pleural thickening	ipsilateral	95	77
	contralateral	19	15
Pleural plaques	ipsilateral	10	8
	contralateral	8	6
Extensive contraction of the chest		17	14
Rib destruction		18	15
Asbestosis		2	2

TABLE II

Diagnostic techniques

Technique	Performed number	Positive for mesothelioma (%)
Pleural fluid cytology	105	63 (60%)
Abrams biopsy	85	58 (68%)
Thoracoscopy	45	39 (87%)
Thoracotomy	21	19 (91%)
Bronchoscopy	3	0 (0%)

The diagnostic procedures used are shown in table 2. Compared to aspiration of pleural fluid and Abrams biopsy, thoracoscopy and thoracotomy were carried out less often, but they gave positive findings in high percentages (87% and 91%, respectively).

Therapy mainly consisted of symptomatic treatment. Recurrent pleural effusions were terminated by drainage. In a number of patients radiotherapy, chemotherapy, surgery or immunotherapy (interferon) were tried. No significant effect on survival was found. Fourteen patients (11%) did not receive any treatment at all. The average survival was 16.5 ± 13 months (median = 11 months). Only five patients were alive 5

years after the diagnosis was made. Figure 1 shows the survival-percentages. The 1-year survival is less than 50% (47%).

Non-narcotic analgetics were able to relief the pain in 63 patients (of the 98 patients given non-narcotic analgetics). When however in a later stage pain became more severe, opiates had to be given in 49 patients. In one patient chordotomy was performed while epidural anaesthesia was given in another patient, both with good results. Eight patients had pain relief after local radiotherapy.

The autopsy findings of 28 patients were available and are listed in table 3. Metastases were most frequent in the liver (60%) and abdominal lymph nodes (38%).

TABLE III

Autopsy-findings of 28 subjects with mesothelioma

metastases in	examined in	positive
diaphragm-ingrowth	26	22 (85%)
pericardium-ingrowth	24	17 (65%)
liver	25	15 (60%)
thoracic lymph nodes	24	13 (54%)
abdominal lymph nodes	26	10 (38%)
kidneys	25	7 (28%)
spine	23	4 (17%)
brain	25	1 (4%)
other	24	11 (46%)

DISCUSSION

In this study we analysed the clinical, diagnostic, and histologic findings in 124 patients with a malignant pleural mesothelioma. The incidence in men is usually several times that in women (13) as is demonstrated in this study. Malignant pleural mesothelioma usually affects people between 45 and 75 years (14). The median age of our subjects at the time of diagnosis was 64.7 years, while 87 patients (78%) were between 50 and 75 years of age. The duration of the asbestos exposure could be calculated from the occupational files of most patients. The median exposure was 33.6 years (range 1-52 yrs.). The latent period was at least 17 years (median 41.2 yrs.), which is in the same range as in other studies (15). The survival from the time of the first symptoms to death was short (median = 11 months). Although some found

different survival times for the various cellular subtypes (16) we did not find significant differences.

In 68 patients (55%) pleural mesotheliomas with a histopathologic diagnosis of mixed type were found. Compared to other series this is rather high. The histopathologic diagnosis of mesothelioma is a difficult one. A strong interobserver variability is a well known phenomenon (17,18). There is a considerable risk of misdiagnosing the mesothelioma for a pulmonary adenocarcinoma if only a small biopsy is examined. Furthermore, in investigating only small portions of tumor, there is considerable risk in missing one component. When large portions of tumor tissue are investigated, the chances of finding both epithelial and connective tissue components are high. Several phenotypic markers have been studied for their efficacy in the differentiation of mesotheliomas from other (metastatic) malignancies, but they are still of only complementary importance (19). We believe that an adequate histopathologic classification can only be reached by examination of a large biopsy, performed by an experienced pathologist. The material should be searched intensively for malignant elements of both epithelial and connective tissue character. Then higher numbers of mixed type will be found. Support for this statement was obtained by comparing the percentages of epithelial or connective tissue type with mixed type in the diagnostic procedures where small portions of tumor (cytology of pleural fluid and Abrams biopsy) or large portions of tumor were obtained (thoracoscopy, thoracotomy, obduction). We found that if only a "small portion- technique" had been performed the mixed type was present in 40% of the patients, whereas in "large portion-techniques" this increased to 73%.

The most frequently found presenting symptoms in our series, dyspnea, pain and weight loss are the usual clinical characteristics of pleural mesothelioma (20,21). A less common symptom is cough. Six patients presented with a vena cava superior syndrome. This means this may not be as infrequent as was suggested by others (22).

Pleural effusions were detected radiologically in no less than 90% of the patients presenting with a malignant mesothelioma. Long term pleural effusions are in 10% due to a malignant mesothelioma (23). Like in our series the pleural effusion usually masks the tumor. Aspiration of the fluid may disclose the malignancy. In this series of patients it was found that asbestos exposure may also give a benign pleural thickening since this sign was found in 15% of the patients on the contralateral side of the thorax. Furthermore, it is obvious that pleural plaques and asbestosis are no premalignant lesions since they were found in a minority of the patients presenting with a malignant pleural mesothelioma and the occurrence was not increased as compared to the generally asbestos exposed population.

Cytology of the pleural fluid was positive for malignant mesothelioma in 60%. An Abrams biopsy could make the diagnosis of mesothelioma definite in 68%. In only

seven cases diagnosis was based on cytology alone. Thoracoscopy and thoracotomy were positive in much higher percentages. This pattern is not unusual (24). In our view the best procedure to be followed is to start with cytology of the pleural fluid, which can easily be obtained together with an Abrams biopsy. If this does not give a definite diagnosis, a thoracoscopy or thoracotomy will be indicated.

The results of several treatment programs are invariably bad (25-27). Patients with a survival of more than two years are only incidentally found. In our study treatment consisted of a mixture of all modalities. Only surgery and immunotherapy seemed to prolong survival, but due to the fact that all sorts of selection-bias can have taken place, this cannot be regarded as a valid statement.

In previous years a number of reports emphasised the frequent occurrence of metastases in patients with a malignant mesothelioma. Metastatic spread does not seem to be linked with histologic type according to some (28), while others found that distant metastases were more frequent in connective tissue type tumors (29). In this study distant metastases were found in mixed type tumours in 13 cases (68%). The numbers of the other histologic types are too small to pronounce upon this matter. The most frequent sites of spread outside the thorax were liver (60%) and abdominal lymph nodes (38%). Compared to other studies these percentages are rather high. The rarity of brain metastases is confirmed (4%)(30).

In conclusion in this series of malignant pleural mesotheliomas a relatively high percentage of mixed types is found. This is probably due to the availability of large portions of tumour specimens for histologic examination. If cytology of the pleural fluid and an Abrams biopsy do not give a definite diagnosis, we recommend that thoracoscopy with biopsies be the diagnostic procedure of choice since this method increases the chance of a positive findings.

ACKNOWLEDGMENTS

We thank Dr. P.H. de Beer for his advice and Mrs. Jacqueline de Goey-van Dooren for typing the manuscript.

REFERENCES

1. Wagner JC, Sleggs CA, Marchand P. Diffuse pleural mesotheliomas and asbestos exposure in the Northwestern Cape Province. Br J Ind Med 1960,17,260-271.
2. Becklake MR. Exposure to asbestos and human disease. N Engl J Med 1982,306,1480-1482.
3. Becklake MR. Asbestos-related diseases of the lung and other organs : their epidemiology and implications for clinical practice. Am Rev Resp Dis 1976,114,187-227.
4. Botha JL, Irwig LM, Strebel PM. Excess mortality from stomach cancer, lung cancer and asbestosis and/or mesothelioma in crocidolite mining districts in South Africa. Am J Epidemiol 1986,123,30-40.
5. Berry G, Newhouse ML, Antonis P. Combined effect of asbestos and smoking on mortality from lung cancer and mesothelioma in factory workers. Brit J Ind Med 1985,42,12-18.
6. Gardner MJ, Acheson ED, Winter PD. Mortality from mesothelioma of the pleura during 1968-78 in England and Wales. Br J Cancer 1982,46,81-88.

7. Archer VE, Rom WN. Trends in mortality of diffuse malignant mesothelioma of the pleura. Lancet 1983,ii,112-113.
8. Connelly RR, Spirtas R, Myers MH, Percy CL, Fraumeni JF. Demographic Patterns for mesothelioma in the United States. J Natl Cancer Inst 1987,78,1053-1060.
9. McDonald JC, McDonald AD. Epidemiology of mesothelioma from estimated incidence. Prev Med 1977,6,426-446.
10. Stumphius J. Epidemiology of mesothelioma on Walcheren Island. Brit J Ind Med 1971,28,59-66.
11. Planteydt HT. Netherlands mesothelioma register. Ann N Y Acad Sc 1979,330,467-472.
12. Commission of the European Communities. Colour atlas of mesothelioma. Jones JSP, Lund C, Planteydt HT, Lancaster, England, 1985.
13. Jones B, Thomas P. Mesothelioma registry data. Lancet 1986,ii,167.
14. Jones B, Thomas P. Incidence of mesothelioma in Britain. Lancet 1986,i,1275.
15. Selikoff IJ, Hammond EC, Seidman H. Latency of asbestos disease among insulation workers in the United States and Canada. Cancer 1980,46,2736-2740.
16. Hillerdal G. Malignant mesothelioma 1982: review of 4710 published cases. Br J Dis Chest 1983,77,321-43.
17. Wright WE, Sherwin RP, Dickson EA, Bernstein L, Fromm JB, Henderson BE. Malignant mesothelioma : incidence, asbestos exposure, and reclassification of histopathology. Brit J Ind Med 1984,41,39-45.
18. Planteydt HT. Observer variation and reliability of the histopathological diagnosis of mesothelioma. Ann N Y Acad Sc 1979,330,761-764.
19. Lee I, Radosevich JA, Chejfec G, Ma Y, Warren WH, Rosen ST, Gould VE. Malignant mesothelioma. Improved differential diagnosis from lung adenocarcinomas using monoclonal antibodies 44-3A6 and 624A12. Am J Pathol 1986,123,497-507.
20. Antman KH. Clinical presentation and natural history of benign and malignant mesothelioma. Semin Oncol 1981,8,313-20.
21. Elmes PC, Simpson MJC. The clinical aspects of mesothelioma. J Med 1976,179,427-449.
22. Ragalie GF, Varkey B, Choi H. Malignant pleural mesothelioma presenting as superior vena cava syndrome. Can Med Assoc J 1983,128,689-691.
23. Martensson G, Hagmar B, Zettergren L. Diagnosis and prognosis in malignant pleural mesothelioma: a prospective study. Eur J Respir Dis 1984,65,169-178.
24. Law MR, Hodson ME, Turner-Warwick M. Malignant mesothelioma of the pleura: clinical aspects and symptomatic treatment. Eur J Resp Dis 1984,65,162-168.
25. McCormack PM, Nagasaki F, Hilaris BS, Martini N. Surgical treatment of pleural mesothelioma. J Thorac Cardiovasc Surg 1982,84,834-842.
26. Antman KH, Blum RH, Greenberger JS, Flowerdew G, Skarin AT, Canellos GP. Multimodality therapy for malignant mesothelioma based on a study of natural history. Am J Med 1980,68,356-362.
27. Harvey VJ, Slevin JL, Ponder BAJ, Blackshaw AJ, Wrigley PFM. Chemotherapy of diffuse malignant mesothelioma. Cancer 1984,54,961-964.
28. Huncharek M, Muscat J. Metastases in diffuse pleural mesothelioma: Influence of histological type. Thorax 1987,42,897-898.
29. Law MR, Hodson ME, Heard BE. Malignant mesothelioma of the pleura: relationship between histological type and clinical behaviour. Thorax 1982,37,810-815.
30. Kaye JA, Wang A, Joachim CL, Seltzer SE, Cibas E, Skarin A, Antman KH. Malignant mesothelioma with brain metastases. Am J Med 1986,80,95-97.

CHAPTER 5

DIAGNOSIS OF HUMAN MALIGNANT MESOTHELIOMA

5.1 Expression of epithelial membrane antigen on malignant mesothelioma cells.

5.2 Cytogenetic analysis of malignant mesothelioma.

CHAPTER 5.1

EXPRESSION OF EPITHELIAL MEMBRANE ANTIGEN ON MALIGNANT MESOTHELIOMA CELLS

An immunocytolochemical and immunoelectron microscopic study

Theodorus H. van der Kwast[1], Marjan A. Versnel[2], Mick Delahaye[3], Anton de Jong[3], Pieter E. Zondervan[3] and Henk Hoogsteden[4]

[1]Department of Patholoy and [2]Department of Cell Biology, Immunology and Genetics, Erasmus University, Rotterdam, [3]Department of Pathology and [4]Department of Lung Diseases, Academic Hospital Dijkzigt Rotterdam, The Netherlands

Acta Cytologica 32, 169-174, 1988.

ABSTRACT

The presence of epithelial membrane antigen (EMA) on malignant mesothelial cells found in pleural and ascitic fluids was demonstrated immunocytochemically using a monoclonal anti-EMA antibody. Serous fluids of 25 patients with malignant mesotheliomas were investigated. In 23 cases, varying numbers of EMA-positive tumor cells were present; in 2 cases, no such cells were found. Immunoelectron microscopy was performed both on Lowicryl-embedded sediments of serous fluids and by application of pre-embedding techniques using the immunogold method. Expression of EMA by the immunogold method was found selectively on the villi of the malignant mesothelioma cells whereas the nonvillous, flat surfaces were largely EMA-negative. The results indicate that immunoelectron microscopy may offer a useful adjunct in the diagnosis of malignant mesothelioma in serous fluids.

INTRODUCTION

The cytologic diagnosis of malignant mesothelioma is hampered by the lack of clear morphologic criteria differentiating benign from malignant mesothelial cells and malignant mesothelial cells from adenocarcinoma cells. Histochemical staining techniques are often applied to reach a higher level of diagnostic accuracy, but even this approach is often found to be inconclusive (9,20). Morphometry has also been employed to improve diagnostic sensitivity, but this technique is not able to distinguish malignant mesothelioma from adenocarcinoma (8).

Electron microscopy (EM) permits the identification of subcellular features specific for mesothelial cells, distinguishing them from adenocarcinoma cells. Characteristics specific for malignancy, however, are generally not detectable using this latter technique (12,19). Therefore, EM alone is not sufficient for the conclusive diagnosis of malignant mesothelioma (20).

The recent advance of immunocytochemistry in cytologic diagnostics has been reflected in a large number of papers describing the use of (monoclonal) antibodies directed against a vast array of antigens (3,11,15). For instance, epithelial membrane antigen (EMA) has been reported to be diagnostic in pleural and ascitic fluids on the basis of its presence on (malignant) epithelial cells and its absence from reactive mesothelial cells (3,7). Regarding the expression of EMA on malignant mesothelial cells, controversial data have been reported (2,10,13). In this paper, we demonstrate the presence of EMA on malignant mesothelial cells in the majority of cases of malignant mesothelioma. In addition, the ultrastructural localization of EMA on these

cells is shown.

MATERIALS AND METHODS

Various fluids were assayed for the presence of EMA-positive cells: 56 cytologically negative serous fluids containing varying numbers of reactive mesothelial cells, 39 cytologically positive serous fluids, pleural fluids from 23 cases of malignant mesothelioma and ascitic fluids from another 2 cases. In all cases, the clinical diagnosis of malignant mesothelioma was confirmed by histology and/or EM. Histochemical staining reactions performed on these serous fluids were alsways consistent with the diagnosis of malignant mesothelioma.

Pleural and ascitic fluids were obtained by puncture; the fluid was centrifuged and the sediment was smeared on glass slides. The smear was allowed to air dry and was then stored in a dry place. After a maximum period of two weeks, the slides were stained for EMA using a monoclonal anti-EMA antibody, obtained from Dako (Copenhagen, Denmark), in a dilution of 1:20 in phosphate-buffered saline (PBS) (0.4 M; pH 7.4) containing 0.05% gelatin and 0.1% NaN_3. This monoclonal antibody was previously referred to as E29 (4). Before adding the primary antibody, the smears were fixed and endogenous peroxidase activity was simultaneously blocked by incubation in methanol containing 3% hydrogen peroxide for 30 minutes. The slides were then overlaid with the anti-EMA antibody for 1 hour, rinsed for 10 minutes in two changes of PBS and incubated for another 30 minutes with a horseradish peroxidase-conjugated rabbit anti-mouse (RAM-PO) antibody (Dako) diluted 1:50 in PBS containing 5% nonimmune human serum and 5% nonimmune rabbit serum. Antibody localization was visualized by incubation of the slides with a TRIS-buffered saline solution (0.05 M; pH 7.4) containing 50 mg % of 3,3 diaminobenzidine tetrahydrochloride (Fluka, West Germany) and 0.03% hydrogen peroxide. Slides were then washed with running tap water, counterstained with hematoxylin and mounted in Malinol. All incubations were performed at room temperature.

For preembedding for immunoelectron microscopy, the sediments of serous fluids were fixed in buffered paraformaldehyde (1.5%) and glutaraldehyde (0.1%) in PBS (pH 7.4) for 24 hours and then stored in PBS contatining 1% paraformaldehyde and 0.1-M sucrose until immunostaining. For immunogold staining, the pellet was washed twice in PBS and then incubated with anti-EMA (1:20) for one hour. The cell suspension was washed twice again in PBS, and the pellet was suspended in a dilution of 10-nm colloidal gold-labeled goat anti-mouse serum (Janssen, Belgium) diluted 1:10 in PBS. After incubation for one hour, the cells were centrifuged again and washed in

PBS. The final pellet was postfixed in 1% w/v OsO_4 in 0.1-M phosphate buffer (pH 7.3) for 12 hours at 4°C, rinsed in the same buffer, acetone dehydrated and Epon embedded for routine transmission electron microscopy (TEM).

For postembedding imunoelectron microscopy, the sediments of serous fluids were fixes in 1.5% v/v paraformaldehyde plus 0.1% glutaraldehyde in 0.1-M phosphate buffer (pH 7.2) at 4°C for four hours. Pellets were then transferred into 0.2% w/v agar in PBS solution kept fluid at 40°C. The pellet was brought into suspension by gentle pipetting. After cooling, the clotted material was stored in a sucrose buffer of 1-M sucrose in 1.1-M phosphate buffer (pH 7.2) with 1% w/v paraformaldehyde at 4°C. Subsequently, the pellets were dehydrated in graded ethanol while temperature was progessively lowered. Finally, the material was infiltrated with Lowicryl K4M-alcohol mixtures and pure catalyzed Lowicryl at -35°C. Polymerization took place under ultraviolet light at -40°C for 24 hours. From the Lowicryl-embedded material, 1-μm-thick sections were cut with a glass knife and stained with toluidine blue to select appropriate areas for ultrathin sectioning. The ultrathin Lowicryl-embedded sections were collected on carbon-coated Formvar filmed mesh 100 copper grids.

The immunologic methods for visualization of the mouse monoclonal anti-EMA antibodies bound to the antigenic sites were those described by Geuze et al. (5).

RESULTS

Three of the 56 smears without cytologic evidence of malignancy contained sporadic EMA-positive cells (Table 1). Follow-up revealed the presence of numerous malignant epithelial cells in one of these three cases one month later. The other two cases were lost to follow-up, but these patients were known to have a metastasized

TABLE I

Presence of EMA-positive cells in cytologically positive and negative serous fluids

	EMA staining		
Cytology*	Negative	Positive	Total
Negative	53	3	56
Positive	1	38	39

* Negative cytology signifies the absence of malignant cells in smears of serous fluids while positive cytology indicates the unequivocal presence of malignant epithelial cells.

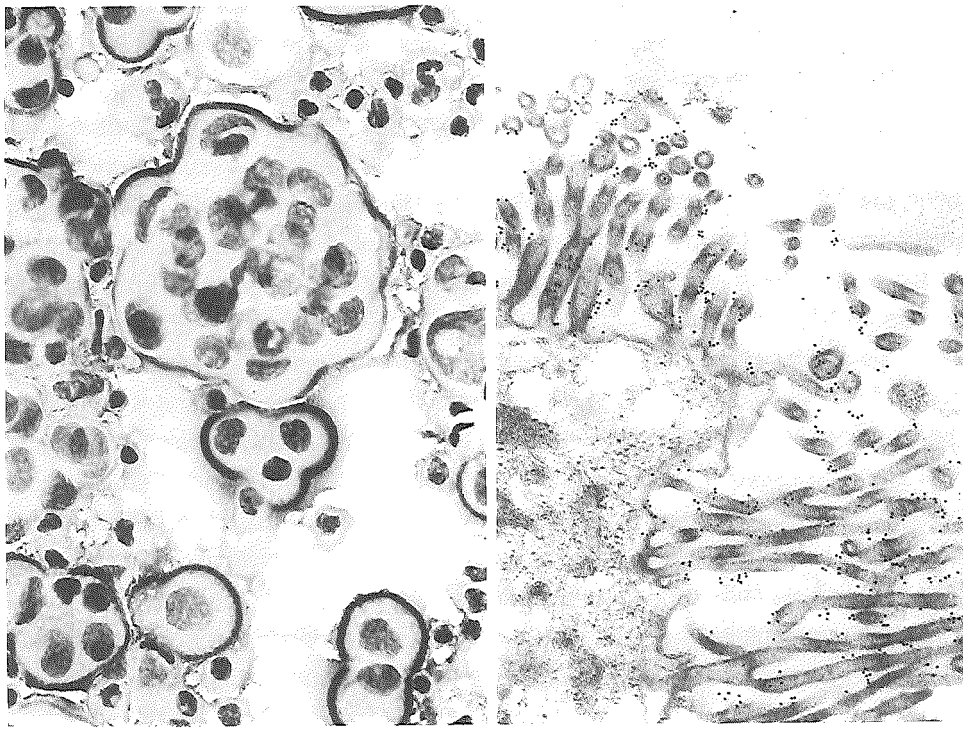

Figure 1. *(left) Cluster of malignant mesothelioma cells stained for EMA. The periphery of the cells shows intense immunoperoxidase staining (x 250).*

Figure 2. *(right) Lowicryl-embedded malignant mesothelioma cell. Postembedding immunogold labeling for EMA shows a discontinuous labeling pattern of the villous surface (x 45,000).*

adenocarcinoma. In 38 of the 39 smears with overt epithelial malignancy, a high proportion of tumor cells stained for EMA.

Twenty-three of the 25 smears of patients with malignant mesothelioma contained varying numbers of moderately to intensely EMA-stained cells (Table II). Staining was observed mainly at the periphery of the cells; at higher magnification, heavily stained hairy protrusions of the membrane could be noted (Figure 1). Some cells stained fully at the circumference while other cells stained only partially. Clusters of cells and so-called "morules" only stained at the periphery. No staining was observed at the inner cell boundaries of these aggregates. In two cases, no EMA staining of the mesothelioma cells was observed.

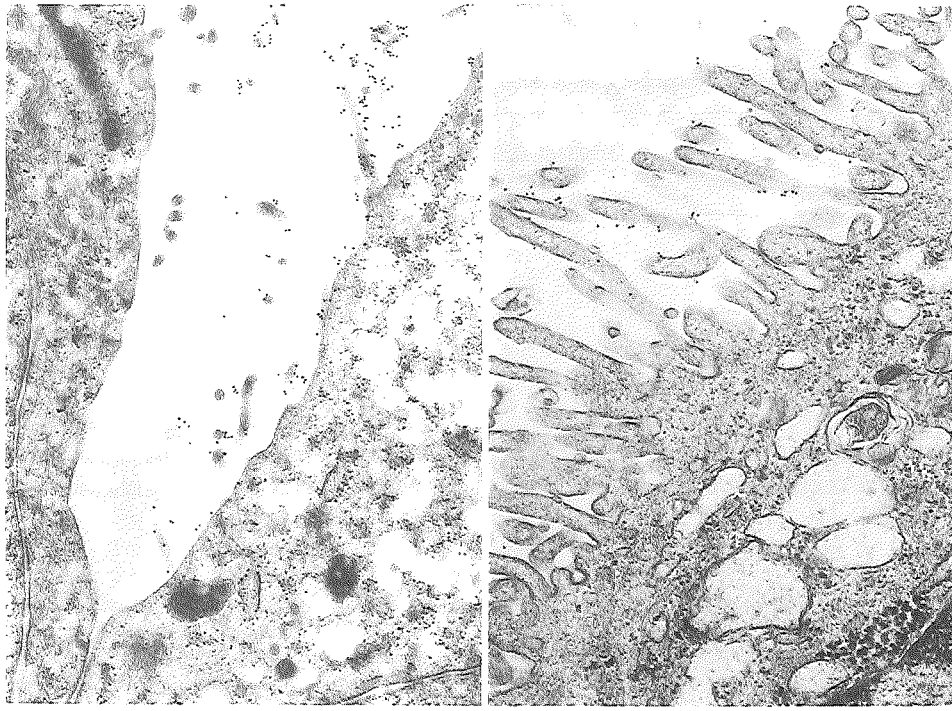

Figure 3. *(left) Lowicryl-embedded malignant mesothelioma cell. Postembedding immunogold labeling for EMA shows numerous gold granules along the surface membrane of the long villi. Notice the absence of immunogold staining of the nonvillous surface and the cytoplasm (x 26,000).*

Figure 4. *(right) Epon-embedded malignant mesothelioma cell labeled for EMA using the preembedding immunogold technique. Glycogen granules (arrow) are clearly visible (x 30,000).*

Immunoelectron microscopy on Lowicryl-embedded sediments of serous fluids containing malignant mesothelioma cells showed gold granules predominantly situatedat the cytoplasmic outer membrane of the long villi of cells with the ultrastructural features of mesothelial cells. The staining for EMA revealed a remarkable, discontinuous pattern (Figure 2). The nonvillous surfaces of these malignant mesothelial cells only sporadically showed gold particles (Figure 3). Similarly, no labeling was found at contiguous borders of cells situated in clusters or morules. No significant numbers of gold granules were present in the cytoplasm of these cells. Preembedding immunoelectron mmicroscopy using either the immunogold or the

TABLE II

Frequency of EMA-positive malignant mesothelioma cells in serous fluids

% EMA-positive cells	Cytologic pattern (no. of fluids)			Total
	Clusters*	Solitary**	Combined***	
0	1	1	0	2
<5	1	0	2	3
5-50	3	2	3	8
>50	6	4	2	12

* Smears containing malignant mesothelioma cells predominantly arranged in clusters, morules or papillary groups, ** Smears containing malignant mesothelioma cells predominantly arranged as single cells or small clusters of two to three cells, *** Smears containing both clusters and single cells.

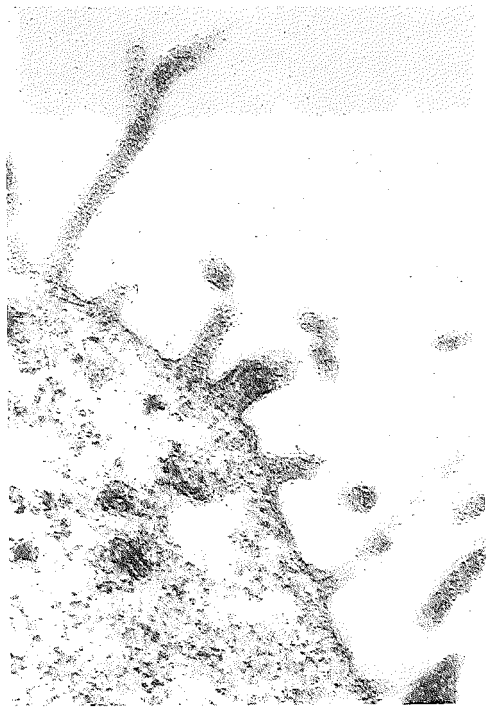

Figure 5. Lowicryl-embedded reactive mesothelial cell. Postembedding immunogold labeling for EMA did not lead to deposits of gold granules on this cell (x 60,000).

immunoperoxidase method yielded essentially the same results. In the Epon-embedded immunostained preparations, the slender, branched villi as well as the presence of glycogen granules, fat droplets, the occasionally extensive intermediate filament pattern and desmosomes could be identified easily, indicating the mesothelial differentiation of the immunostained cells (Figure 4). Preparations incubated with nonrelevant monoclonal antibodies (anti-T3 or anti-leukocyte-common antigen) did not show labeling of mesothelial cells.

In addition, postembedding immunoelectron microscopy was performed on two pleural fluids containing large numbers of reactive mesothelial cells. Cells with the ultrastructural features of mesothelial cells were not stained, even though large numbers of long, slender villi were projecting from their surfaces (Figure 5).

DISCUSSION

The presence of EMA on malignant mesothelial cells was found in the majority of cases. These findings confirm an earlier study on a few cases of malignant mesotheliomas. To et al., (18), using a polyclonal anti-EMA antiserum, found one of two cases to be positive. In contrast, Battifora (1) found the only cytologic case he tested to be negative. Staining for human milk fat globule (HMFG) glycoproteins or EMA on paraffin-embedded tumorous mesothelioma tissue has yielded contradictory data. Some authors reported that anti-EMA can distinghuish between adenocarcinomas and malignant mesotheliomas since no staining of the latter was observed (2). Other authors have been able to demonstrate the presence of EMA on histologic specimens of malignant mesothelioma (10,13,14). The use of different batches of anti-EMA antibodies might partially explain these discrepant findings. Battifora and Kopinski (2), using the anti-HMFG2 antibody, failed to achieve immunostaining in all 28 malignant mesothelioma tested while Pinkus and Kurtin (13), using the same monoclonal antibody as applied in our study, found 5 of 6 pleural mesotheliomas to be immunopositive.

In the series of cytologically negative smears tested in this study, it appeared that reactive mesothelial cells did not express EMA, although a few EMA-positive cells were present in three cases (Table 1). In one of these three cases, however, the pleural fluid contained numerous malignant epithelial cells one month later. A few previous papers have indicated the occasional presence of HMFG-2/EMA-positive reactive mesothelial cells in serous effusions of a small proportion of patients without known malignancy (6,17). The clinical significance of the occurrence of EMA-positive benign mesothelial cells is not known. Apparently, EMA may not serve as a fully reliable indicator of malignancy, though its sensitivity must still be considered high. Thus far, no onther marker for mesothelial malignancy has been described. Neither carcinoembryonic antigen nor the antigen detected by the recently described monoclonal antibody B72.3 is present on malignant mesothelioma cells (16,21).

Electron microscopy has been introduced as an ancillary test in the differential diagnosis of malignant mesothelioma, and the ultrastructural characteristics of mesothelial cells are now well documented (19). Although epithelial and mesothelial

malignant cells can often be distinghuished on the basis of ultrastructural characteristics, the value of EM in distinguishing between benign and malignant mesothelial cells is doubtful. Using EMA as a specific and sensitive marker of malignancy, immunoelectron microscopy was applied in this study to differentiate between reactive and malignant mesothelial cells on the one hand and between mesothelial and epithelial cells on the other hand. Preembedding immunoelectron microscopy gave excellent morphologic results combined with intense immunogold or immunoperoxidase labeling. Postembedding techniques showed that immunoreactive material was nearly exclusively present on the outher surface of the malignant mesothelioma cells. The preferential staining of the villi of these mesothelioma cells suggests that, in malignant mesothelioma, the appearance of these villi is strongly associated with EMA expression. In contrast, the villi of reactive mesothelial cells were not labeled at all. One could therefore consider the expression of EMA on malignant mesothelial cells as a differentiation-associated phenomenon. Additionally, it was striking that EMA was nearly entirely absent from the cytoplasm of the malignant mesothelial cells in the six case studied.

In conclusion, this study indicates that immunoelectron microscopy on preparations of serous fluids suspected as being from malignant mesothelioma may be helpful in making the diagnosis.

ACKNOWLEDGEMENTS

The authors thank Prof.dr. A. Dekker and Dr. D.I. Blonk for helpful advice, Mr. C.C.J. van Vroonhoven for technical support and Ms M. Hanegraaff for secretarial assistance.

REFERENCES

1. Battifora H: Recent progress in the immunohistochemistry of solid tumors. Semin Diagn Pathol 1:251-271, 1984.
2. Battifora H, Kopinski MT: Distinction of mesothelioma from adenocarcinoma: An immunohistochemical approach. Cancer 55:1679-1685, 1985.
3. Epenetos AA, Canti G, Taylor-Papadimitriou J, Curling M, Bodner WF: Use of two epithelium-specific monoclonal antibodies for diagnosis of malignancy in serous effusions. Lancet 2:1004-1006, 1982.
4. Gatter KC, Alcock C, Herget A, Pulford KA, Heyderman E, Taylor-Papadimitriou J, Stein H, Mason DY: The differential diagnosis of routinely processed anaplastic tumors using monoclonal antibodies. Am J Clin Pathol 82:33-43, 1984.
5. Geuze HJ, Slot JW, Van der Ley PA, Scheffer RCT, Griffith JM: Use of coloidal gold particles in double labeling immunoelectron microscopy of ultrathin frozen tissue sections. J Cell Biol 889:653-655, 1981.
6. Ghosh AK, Mason DY, Spriggs AI: Immunocytochemical staining with monoclonal antibodies in cytologically "negative" serous effusions from patients with malignant disease. J Clin Pathol 36:1150-1152, 1983.
7. Hilborne LH, Cheng L, Nieberg RK, Lewin KJ: Evaluation of an antibody to human milk fat globule antigen in the detection of metastatic carcinoma in pleural, pericardial and peritoneal fluids. Acta Cytol 30:245-250, 1986.
8. Kwee WS, Veldhuizen RW, Alons CL, Morawetz F, Boon ME: Quantitative and qualitative differences between benign and malignant mesothelial cells in pleural fluid. Acta Cytol 26:401-406, 1982.
9. Kwee WS, Veldhuizen RW, Golding RP, Mullink H, Stam J, Donner R, Boon ME: Histologic distinction

between malignant mesothelioma, benign pleural lesion and carcinoma metastasis. Virch Arch (Pathol Anat) 397:287-299, 1982.
10. Marshall RJ, Herbert A, Braye SG, Jones DB: Use of antibodies to carcinoembryonic antigen and human milk fat globule to distinguish carcinoma, mesothelioma, and reactive mesothelium. J. Clin Pathol 37:1215-1221, 1984.
11. Ménard S, Rilke F, Della Torre G, Mariani-Constantini R, Regazzoni M, Tagliabue E, Alasio L, Colnaghi MI: Sensitivity enhancement of the cytologic detection of cancer cells in effusions by monoclonal antibodies Am J Clin Pathol 83:571-576, 1985.
12. Murad TM: Electron microscopic studies of cells in pleural and peritoneal effusions. Acta Cytol 17:401-409, 1973.
13. Pinkus GS, Kurtin PJ: Epithelial membrane antigen: A diagnostic discriminant in surgical pathology: Immunohistochemical profile in epithelial, mesenchymal and hematopoietic neoplasms using paraffin sections and monoclonal antibodies. Hum Pathol 16:929-940, 1985.
14. Sloane JP, Hughes F, Ormerod MG: An assessment of the value of epithelial membrane antigen and other epithelial markers in solving diagnostic problems in tumour histopathology. Histochem J 15:645-654, 1983.
15. Szpak A, Johnston WW, Lottich SC, Kufe D, Thor A, Schlom J: Patterns of reactivity of four novel monoclonal antibodies (B72.3, DF3, B1.1 and B6.2) with cells in human malignant and benign effusions. Acta Cytol 28:356-367, 1984.
16. Szpak CA, Johnston WW, Roggli V, Kolbeck J, Lottich C, Vollmer R, Thor A, Schlom J: The diagnostic distinction between malignant mesothelioma of the pleura and adenocarcinoma of the lung as defined by a monoclonal antibody (B72.3). Am J Pathol 122:252-260, 1986.
17. To A, Coleman DV, Dearnaley DP, Ormerod MG, Stelle K, Neville AM: Use of antisera to epithelial membrane antigen for the cytodiagnosis of malignancy in serous effusions. J Clin Pathol 34:1326-1332, 1981.
18. To A, Dearnaley DP, Ormerod MG, Canti G, Coleman DV: Epithelial membrane antigen: Its use in the diagnosis of malignancy in serous effusions. Am J Clin Pathol 78:214-219, 1982.
19. Warhol MJ, Hickey WF, Corson JM: Malignant mesothelioma: Ultrastructural distinction from adenocarcinoma. Am J Surg Pathol 6:307-314, 1982.
20. Whitaker D, Shilkin KB: Diagnosis of pleural malignant mesothelioma in life: A practical approach. J Pathol 143:147-175, 1984.
21. Whitaker D, Sterrett GF, Shilkin KB: Detection of tissue CEA-like substance as aid in the differential diagnosis of malignant mesothelioma. Pathology 14:255-258, 1982.

CHAPTER 5.2

CYTOGENETIC ANALYSIS OF MALIGNANT MESOTHELIOMA

A. Hagemeijer, M.A. Versnel*, E. Van Drunen, M. Moret, M.J. Bouts*, Th.H. van der Kwast** and H.C. Hoogsteden***

Dept. of Cell Biology and Genetics, *Dept. of Immunology, **Dept. of Pathology, Erasmus University, Rotterdam and ***Dept. of Pulmonary Disease, Academic Hospital Dijkzigt, Rotterdam, The Netherlands

Cancer Genetics and Cytogenetics, in press.

ABSTRACT

Cytogenetic analyses of 40 confirmed malignant mesotheliomas are reported. Pleural effusion cells were studied in 90% of the cases by the direct method and/or after culture. Biopsy and ascites fluid were also analyzed in some patients. A normal karyotype was found in 9 cases and complex karyotypic abnormalities were observed in 30 cases. In one case, analysable metaphases were not obtained. The chromosomal changes were all complex and heterogeneous; no consistent, presumably specific abnormality was detected. Nevertheless two main patterns of non-random abnormalities were seen i.e. 1) loss of chromosomes no. 4 and 22, 9p and 3p in the large majority of abnormal cases and corresponding to a hypodiploid and/or hypotetraploid modal chromosome number. 2) gain of chromosomes no. 7, 5 and 20 with also deletion or rearrangement of 3p in the hyperdiploid cases, which were a minority in our serie. These findings are discussed in view of other reported cytogenetic studies of malignant mesothelioma, asbestos exposure and possible mechanism of malignant transformation.

INTRODUCTION

Malignant mesothelioma (MM) is a mesodermally derived tumor that occurs with a still increasing frequency, particularly in regions with mining and shipyard industry (1). A history of asbestos exposure is found in at least 80% of the cases (2) and a lag phase of 10 à 40 years between exposure and development of tumor is not unusual. The most frequent localization is the pleura, although primary sites in pericard or peritoneum are not exceptional. In malignant pleural mesothelioma the first symptoms are dyspnea and chest pain. Radiological examination often reveals a pleural effusion. Cytological discrimination between benign inflammatory or reactive effusions, malignant mesothelioma and carcinoma metastases is not always clear (3). The physical and radiological signs also lack specificity and so far sure diagnostic criteria are missing, but most needed.

Cytogenetic analysis of hematological malignancies has shown that particular chromosomal aberration are specifically associated with subtypes of leukemia or lymphoma and that they constitute a diagnostic and prognostic factor independent of the other clinical and pathological considerations (4). In solid tumors also specific chromosomal rearrangements have been described e.g. t(11;22) in Ewing sarcoma or t(12;16) in liposarcoma (5-6). Deletion of specific chromosomal regions have also been associated with particular cancers e.g. del(13)(q14) in retinoblastoma (7) or

del(3)(p21) in small cell carcinoma of the lung (8), findings that suggest the loss of regulator genes as the mechanism of tumorigenesis in these particular malignancies. So far, cytogenetic analysis of malignant mesothelioma using banding techniques has been reported in only a limited number of patients, 2 *in vitro* cell lines and 5 *in vivo* cell lines maintained on nude mice (9-16). These studies showed extensive aneuploidy, preferential involvement of some chromosomes but could not indicate a specific chromosomal rearrangement. The complexity of the karyotypes observed suggest tumor progression with appearance of secondary changes. Furthermore, many cases were studied after that the patient had received cytotoxic therapy or after long period of *in vitro* culture. It is only by studying a large number of primary cases that specific cytogenetic primary changes will be found and their eventual diagnostic and prognostic value disclosed. We report here on cytogenetic studies of 40 cases of newly diagnosed, untreated, malignant mesothelioma. Special care has been taken to include only the cases with very good pathological evidence for the diagnosis of malignant mesothelioma. The karyotypes were studied on pleural effusion cells in most cases and/or from tumor biopsy obtained by thoracoscopy or at autopsy. The abnormal karyotypes showed numerous numerical and structural chromosomal changes. In a number of cases a recurrent pattern of changes seems to emerge that could become of diagnostic value.

MATERIALS AND METHODS

Patients

Patients were referred to us from the departments of Pulmonary Diseases of the Academic Hospital and from the other hospitals in Rotterdam and Vlissingen area. Rotterdam and Vlissingen are large harbors with important ship-building and ship-repair facilities, where asbestos was and is still extensively used. As a consequence, the occurrence of malignant mesothelioma is currently of about 7 cases/100.000 inhabitants/year and still increasing. The overwhelming majority of cases have a history of occupational asbestos exposure of at least a few months duration, including housewives that are getting exposed when dusting off the working cloths of their husbands. Pleural fluid was aspirated and used for cytology and pathological diagnosis, for cytogenetics and for establishing long term cultures. Treatment is only palliative and most patients died at home. But, autopsy was performed on the rare cases dying in hospital. As control, cytogenetic analysis of non-malignant reactive pleural effusions (post-trauma, infections, heart failure) and of metastatic adenocarcinomas were also performed.

Pathological diagnosis of malignant mesothelioma.

Three histological types of MM can be distinguished: Epithelial (E), fibromatous (F) and mixed (M). Epithelial and mixed forms exfoliate malignant cells in effusions, while the fibromatous MM is intraparietal, and does not exfoliate. The latter type, thus, requires biopsy of the tumor for diagnosis and when a pleural exudate is present it is often reactive, lacking malignant cells. Cells present in effusions were analyzed by 1) light microscopy, 2) immunocytochemistry using antibodies specific for carcinoembryonic antigen (CEA) and epithelial membrane antigen (EMA) and 3) electron microscopy. Whenever possible, cytological diagnosis was confirmed by histology of tumor material obtained by needle biopsy, thoracoscopy or at autopsy. For this study we retained only the patients with a diagnosis of malignant mesothelioma established by at least two, preferentially three different techniques i.e. cytology, electron microscopy, histology and/or that passed the review committee. In the Netherlands, the histology of cases suspected of MM are reviewed by the Dutch mesothelioma panel of 3 pathologists.

Cytogenetics

a) Effusions. Pleural fluids of untreated patients were received in sterile plastic tubes containing heparin (5000 units), 5 ml Ham's F10 culture medium with fetal calf serum (15% v/v) and antibiotics, and in some colcemid (1-2 μgr). The samples reached the laboratory within 30 to 40 minutes where they were processed using a direct method (DM). The cells from 1 or 2 tubes containing colcemid were washed with RPMI 1640 culture medium, swelled in KCl 0.075 M and fixed according to standard procedures. Other samples (not containing colcemid) were put into culture and harvested after one to 3 days (short term culture) or after one to 4 weeks (long term cultures). In some cases, permanent cell lines were obtained that were subcloned and karyotyped (17,18). For harvesting of the cultures, the mitotic cells were shaked into the supernatant fluid, collected in tubes containing colcemid, washed once and processed as above. When the shaking method failed to provide enough metaphases, colcemid was added to the cultures for a time varying from 1 hour to overnight. Subsequently the mitotic cells were loosend by shaking of the culture flasks, and harvested as described above. The remaining monolayer was then flooded with KCl-EGTA (19) for 20 minutes, and scrapped out with a rubber policeman, washed with KCl 0.075 M and processed as above. The chromosomes were identified using RFA, QFQ and GTG banding techniques and analyzed according to the ISCN (1985).

b) Biopsy and autopsy tumor material were first cut into pieces and dissociated by overnight treatment with collagenase, then put into culture in Ham's F10 culture medium containing antibiotics, glutamine and 15% fetal calf serum. When enough tumor material was available, additional cultures supplemented with Epidermal Growth Factor (EGF) with or without hydrocortisone (HC) were also set up. Successfully established cultures were karyotyped as described above.

RESULTS

Eighty eight samples from 71 patients were received. Malignant mesothelioma was diagnosed in 40 patients, and was the most probable diagnosis in 8 other patients. Pleural effusions were of non-malignant origin in 14 patients and from metastatic carcinoma in 10 cases. Among the 40 cases of confirmed malignant mesothelioma 4 were female (age 72-83) and 36 were male (age: 44-83) (Table 1). History of asbestos exposure was positive in 35 cases, unknown in 3 cases and probably negative in 2 female patients.

Survival was short: 73% of the patients died within one year of diagnosis, 34% already within 6 months. Only one patient survived beyond the second year. The primary site was the pericard in one patient (Me-21) and the pleura in the other, the right pleura in 60% and left pleura in 40% of the cases. Pathological diagnosis was carefully done on biopsies (Figs. 1a,b) and on cytological samples, including electron microscopy (Figs. 1c,d). Epithelial tumors were found in 17 cases, mixed tumors in 13 cases and fibromatous tumors in 4 cases; in the remaining 6 cases epithelial cells were seen in effusion, but because of absence of histology a mixed type could not be

TABLE 1

Age distribution, asbestos exposure and pathological diagnosis in 36 males and 4 females with malignant mesothelioma

sex		age (years)			asbestos exposure			pathological type			
		44-55	56-70	71-83	+	?	-	E	E*	M	F
Male:	36	8	22	6	33	3	0	16	6	12	2
Female:	4	0	0	4	2	0	2	1		1	2
Total:	40	8	22	10	35	3	2	17	6	13	4

E* epithelial by cytology only.

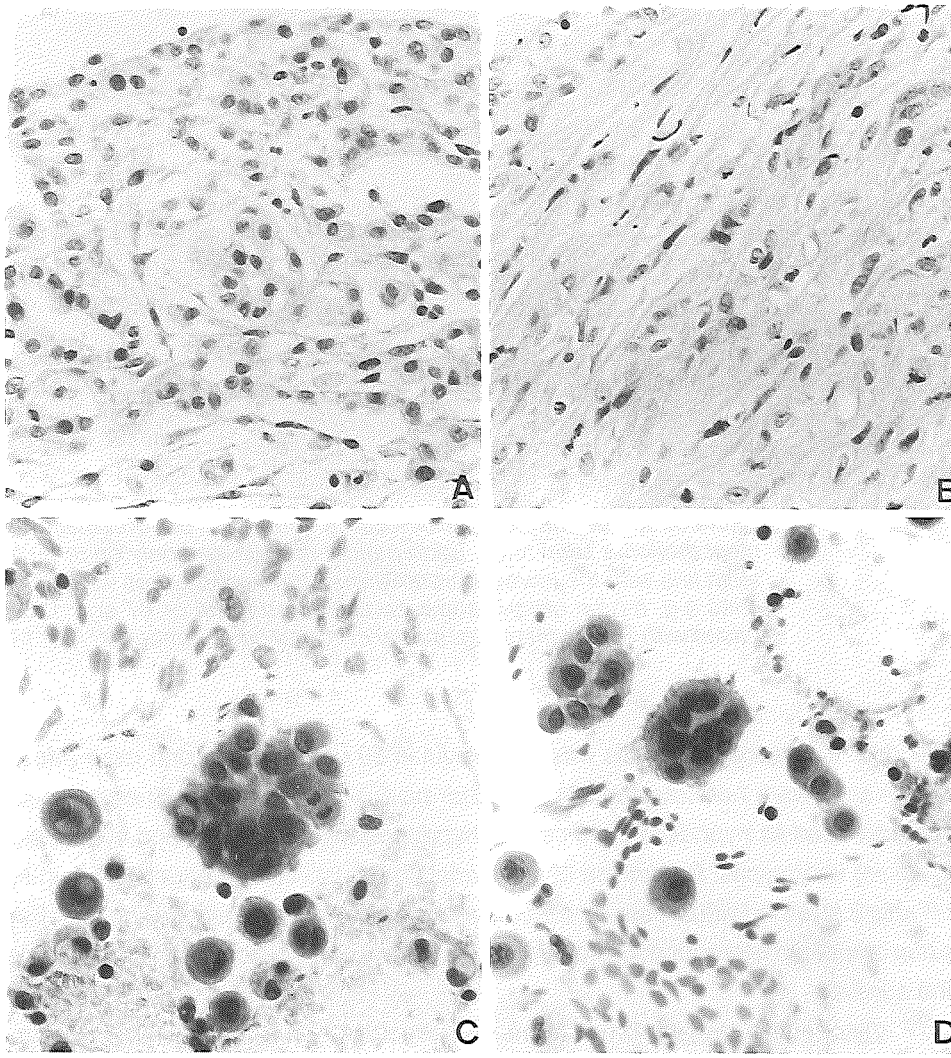

Figure 1. *A: Epithelial growth pattern in a pleural biopsy of mixed type of malignant mesothelioma Me-72 (x250). B: Fibrous growth pattern in the same biopsy as A. (x25O). C and D: Cytological smears of a pleural effusion with several characteristic tumor-cell aggregates and single tumor cells showing cytoplasmic vacuolization (C) and cytoplasmic membrane protrusions (D) (Me-84) (x250).*

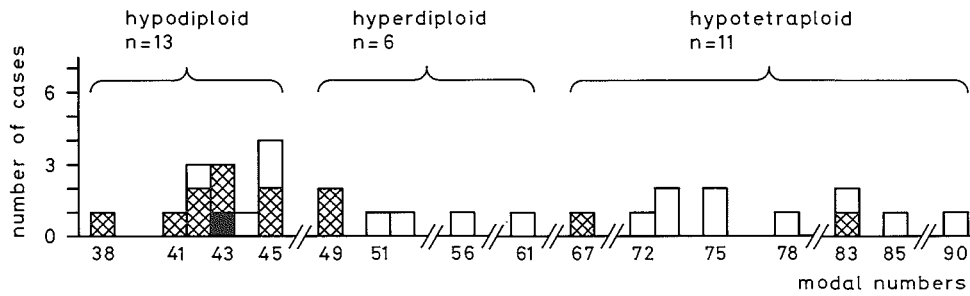

Figure 2. *Distribution of modal chromosome number of 30 malignant mesothelioma stem lines. Each patient is indicated only once by the stem line with the lowest chromosome number. ▩ cases with 2 stem lines e.g. modal chromosome number 42 and 84 or 67 and 135. ■ cases with 3 stem lines e.g. modal chromosome number 43/80/140.*

excluded. Cytogenetic analysis was successful in 39 cases and in one case (Me-45) metaphases were not obtained. Clonal abnormalities were found in 30 cases, which were classified as hypodiploid (13 cases), hypotetraploid (11 cases) and hyperdiploid (6 cases) karyotypes. Repartition of the modal chromosome numbers is given in Fig. 2. Table 2 gives individual data on patients according to the cytogenetic findings. Each group appears heterogeneous regarding age of patients, histological type of tumor, and survival.

Cytogenetic studies. On effusions, the direct method (DM) was most reliable at disclosing abnormal metaphases. The quality of the metaphases was irregular, and not all cells could always be fully karyotyped.

Short term culture improved the quality of metaphases, but often only normal karyotypes were found and it was soon evident that the direct method was required for a valid cytogenetic study. Only a fraction of the samples sustained long term cultures, eventually giving rise to established cell lines (20,21). The long term cultures (1 to 8 weeks) showed a normal karyotype (NN) or a mixture of normal and abnormal cells (AN). The cell lines were subcloned to pure malignant mesothelioma cells with the corresponding abnormal karyotype as reported previously (20). Biopsies were all cultured before karyotyping. In the appendix, the different tissues and technical con-

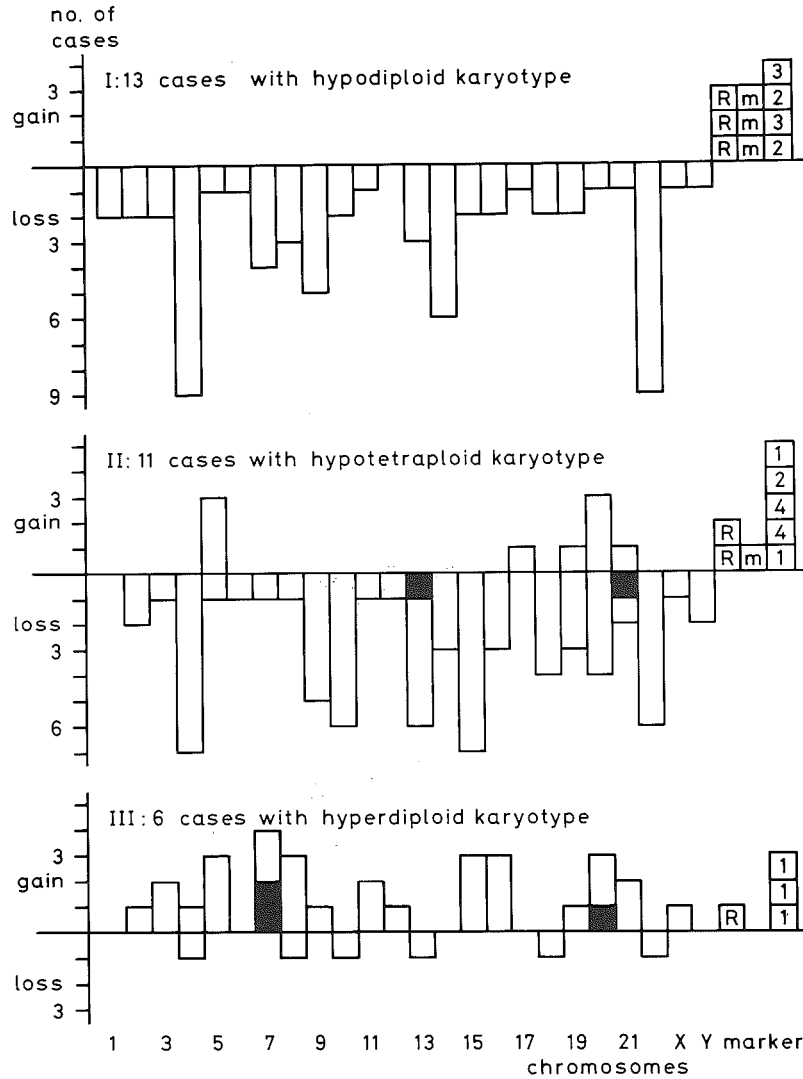

Figure 3. *Histograms showing the numerical changes observed in 30 malignant mesotheliomas with abnormal karyotype. When clonal evolution was seen, the less evolved (simplest) stem line was considered. For distinction between hyperdiploid (III) and hypotetraploid (II), see text. Black boxes indicate loss or gain of 2 copies of a given chromosome in a given stem line. The unidentified markers are also given: R = ring chromosome, m = minute marker, number in open box indicate the average number of markers in a given case.*

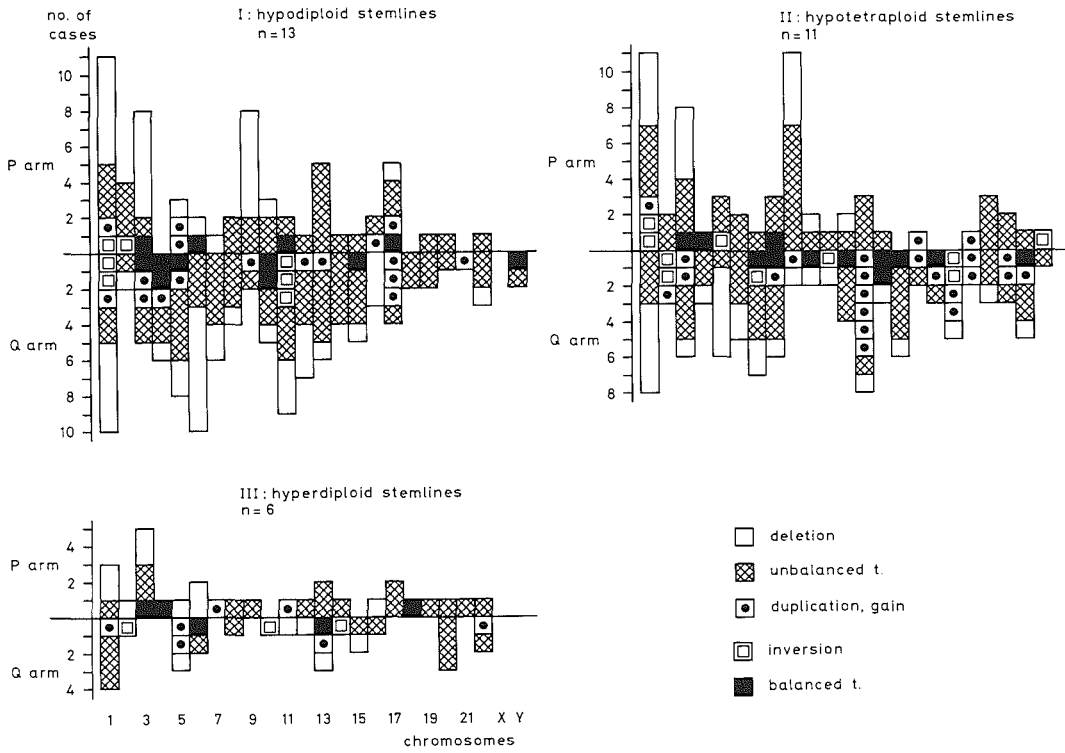

Figure 4. *Histograms showing the involvement of the short and long arm of each chromosome in structural abnormalities. Each marker is represented only once, even if more copies were seen, as was usually the case in the hypotetraploid karyotype. Inversions were paracentric or pericentric, in the latter case they have been indicated on the arm with the more distal breakpoint, gain of chromosomes are either rare duplications, isochromosomes or whole arm translocations like 13q or 17q that were often found in marker formation.*

ditions used for cytogenetic studies are given for each sample together with detailed description of stem line karyotypes. Non-clonal aberrations (random loss of chromosomes and structural changes) were found regularly as well as a tendency to tetraploidization in some samples. In 3 cases (Me-61, Me-77 and Me-95) small clones with loss of Y as only abnormality were observed and not considered as clonal aberrations relevant to malignant mesothelioma.

TABLE 2

Clinical and pathological data of MM patients classified according to cytogenetic findings

patient no.	sex/age at diagnosis	asbestos exposure (1)	survival (Mo) (2)	primary site (3)	histo-logical type (4)	path. criteria (5) cytology	EM	hist.	autopsy
A. 10 Patients with Normal Karyotype of Cytogenetic Failure (Me-45)									
Me-21	M,45	++	6.5	Pericard	E	-		+	
Me-31	M,60	++	1.5	P,l	M	+		+	
Me-51	M,60	++	7	P,l	M	+		+	
Me-57	M,58	+	16	P,r	E	-	+	+	
Me-67	M,66	?	3	P,r	M	+			+
Me-77	M,83	+	20	P,r	M	+	+		+
Me-78	M,71	?	10	P,r	E*	+	+		
Me-86	M,51	++	9	P,r	E	+	+	+	
Me-94	M,61	++	11+	P,l	E	-		+	
Me-45	M,51	+	18	P,r	E	+	+	+	
B. 13 Patients with Hypodiploid Karyotype									
Me-9	M,67	+	20	P,l	E	nd		+	
Me-19	F,75	-	3	P,r	E	+	+	+	
Me-20	M,59	+	2.5	P,l	F	-		+	
Me-26	M,50	++	11	P,l	M	+	+	+	
Me-27	M,67	+	6	P,r	M	+	+	+	
Me-72	M,65	+	12	P,r	M	+		+	
Me-73	M,58	++	11	P,r	M	+	+	+	
Me-83	M,52	++	14	P,l	M	+	+		+
Me-84	M,70	+	4	P,r	E	+	+	+	
Me-85	M,44	+++	14+	P,l	E*	+	+		
Me-100	F,76	-	5	P,l	M	+	+		+
Me-102	M,72	+++	7	P,l	E*	+	+		
Me-105	M,67	++	4	P,r	E	+	+	+	
C. 11 Patients with Hypotetraploid Karyotype									
Me-3	M,70	+	19	P,r	E	+		+	
Me-10	M,67	+	10	P,r	M	+	+	+	
Me-14	M,48	+	5.5	P,l	E	+	+	+	
Me-25	M,78	+	8.5	P,l	E	+	+	+	+
Me-41	M,61	++	2	P,r	E*	+	+		
Me-48	M,62	++	11	P,r	M	-			+
Me-56	M,64	+++	19	P,r	E*	+	+		
Me-64	M,76	+	11	P,r	E	nd		+	+
Me-88	F,72	+++	25+	P,r	F	nd		+	
Me-96	M,46	+	1	P,l	F	nd		+	
Me-104	M,68	+++	14	P,r	E*	+	+		

D. 6 Patients with Hyperdiploid Karyotype

Me-13	M,57	+	6	P,r	E	+	+	+	
Me-50	M,60	?	20+	P,r	E	+	+	+	
Me-61	M,75	+	4	P,l	E	+	+	+	
Me-62	M,64	++	11	P,r	E	+	+	+	
Me-82	F,83	+	1	P,r	F	-			+
Me-95	M,57	++	8	P,l	M	+		+	+

(1) +++ and ++ high exposure (workers in insulation, ship building, demolition, cement factory and direct handling of asbestos), +: worker in potentially exposed environment, ?: no data, (2) Survival from time of clinical diagnosis in months, (3) P = pleura, l = left, r = right, (4) E = Epithelial, M = Mixed, F = Fibromatous, E = Epithelial by cytology only, no biopsy, (5) Cytology on effusion cells, including immunocytology using EMA and CEA specific antibodies, EM = electron microscopy on effusion cells and/or biopsy, nd = no data.

Normal karyotypes were found in 9 patients pleural effusions (Table 2A). In 3 cases (Me-21, Me-57 and Me-94) the pleural fluid contained none or only very few presumably malignant cells and the diagnosis was established histologically. In 2 cases (Me-67 and Me-86) the direct method was not applied. In 4 cases (Me-31, Me-51, Me-77 and Me-78) with pathological fluid, adequate number of cells were analyzed in DM and culture and failed to show clonal abnormalities. Data concerning Me-45 of whom cytogenetic studies failed are also given in Table 2A.

All 30 cases with clonal abnormalities showed complex numerical and structural chromosomal changes. Karyotypic evolution by duplication of the stem line was common and as a consequence most metaphases contained a large number of chromosomes. In these cells secondary changes were also seen, either numerical by random loss of chromosomes or structural by formation of a new (single copy) marker chromosome.

In 13 patients, a hypodiploid stem line was found in at least a few cells. Cells with 75-85 and more than 130 chromosomes were also found as a result of duplication of the hypodiploid stem line. In one patient (Me-84) cytogenetic analyses of one biopsy provided evidence for aneuploidy due to cell fusion of a normal diploid cell with a hypodiploid abnormal cell.

In 11 cases hypotetraploid karyotypes were found with modal chromosome numbers varying from 67 to 90. Analysis of the abnormalities showed similarities with the findings in hypodiploid cases suggesting duplication of an earlier (and not observed) hypodiploid stem line. Also, 2 copies were found of most rearranged chromosomes.

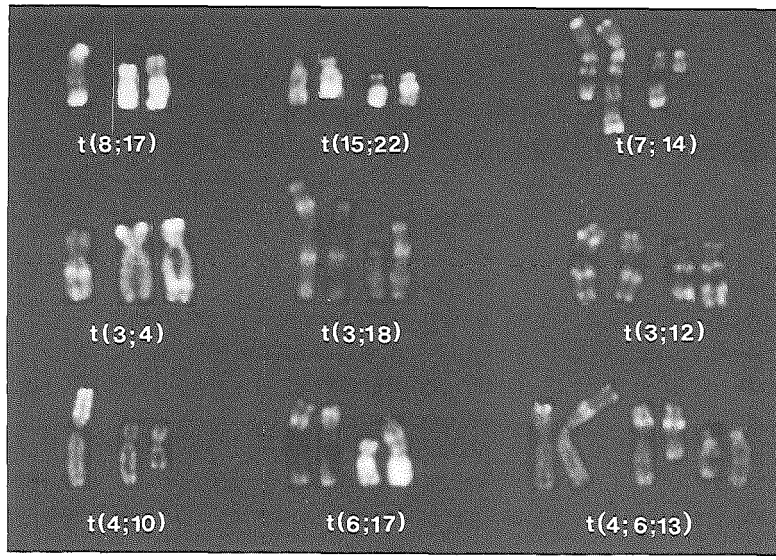

Figure 5. *Examples of balanced translocations observed in MM karyotypes and described in Table 3. These are t(8;17) and t(15;22) in Me-41, t(7;14) in Me-104, t(3;4) in Me-100, t(3;18) in Me-95, t(3;12) in Me-56, t(4;10) in Me-48, t(6;17) in Me-83 and t(4;6;13) in Me-50. In each chromosomal pair, the normal homologue is on the left, and missing in 3 instances.*

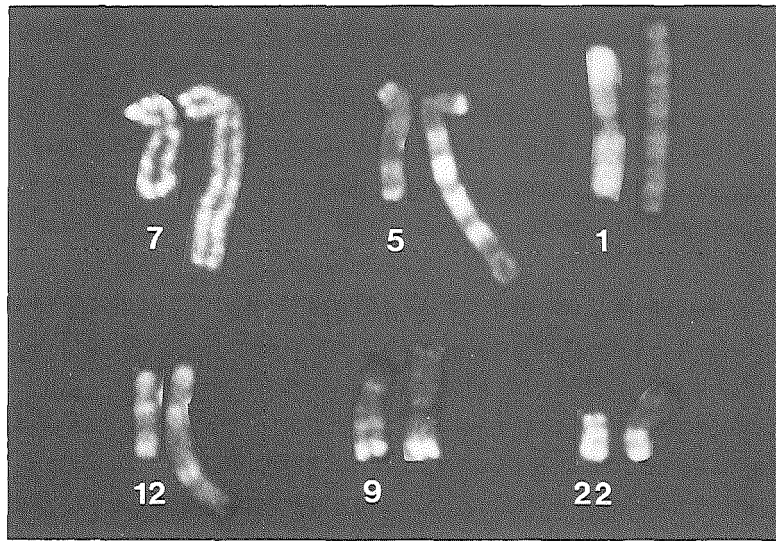

Figure 6. *Marker chromosomes with stretch of unidentified chromosomal material, presumably HSR: 7q+ in Me-9, del(5)(q11q22)q+ in Me-48, dup 1q? in Me-96, 12q+ in Me-73, 9p+ and 22p+ in Me-102. The normal homologue is on the left. R-bands with acridine orange.*

TABLE 3

Balanced translocations observed in 11 cases of malignant mesothelioma

case no.	translocations
Me-9	t(4;10)(q24;q23)
Me-20	t(3;10)(q22;q22)
Me-26	t(Y;15)(q12;q14 or q15),t(5;11)(q35;p13)
Me-41	t(8;17)(p11;q11),t(15;22)(q21;q11)
Me-48	t(4;10)(p11;q11) or (q11;p11)
Me-50	t(4;6;13)(p16;q14;q13)
Me-56	t(3;12)(p21;q23)
Me-83	t(6;17)(p21;p12)
Me-95	t(3;18)(p14;p11)
Me-100	t(3;4)(p21;q31)
Me-104	t(7;14)(q35;q12)

In 6 cases with an hyperdiploid karyotype (modal chromosome number from 49 to 61), the pattern of changes appeared different.

Numerical chromosome aberrations

Gains and losses of chromosomes are plotted on 3 histograms (Fig. 3). In cases with multiple stem lines by duplication the modal karyotype with the lowest chromosome number was used. The hypotetraploid cells were analyzed using the tetraploid karyotype as reference and allowing for random loss: thus 1 or 2 copies of a chromosome was registered as a loss, 3 or 4 copies as normal and 5 or more copies as gain.

Not unexpectedly, the hypodiploid and hypotetraploid karyotypes showed a similar profile with non-random losses of chromosomes 4 (16x) and 22 (15x) followed by losses of # 9 (10x), # 10 (8x) and chromosomes of the D-group.
In the hyperdiploid karyotypes, non-random gain of chromosomes 7 and 20 were seen, followed by trisomy 5, 8, 15 and 16 in half of the cases.

Gain of 5 and 20 was also seen in 3 hypotetraploid karyotypes, concurrently (Me-3, Me-88 and Me-96). This suggests that gain of 5 and 20 are either secondary changes or that these 3 cases, despite their high modal chromosomal number, were misclassified and should have been included in the hyperdiploid category. Remarkably, also, two of these cases were of the fibromatous type.

TABLE 4

Marker chromosomes with possible HSR

patient no.	marker chromosomes
Me-9	7q+
Me-48	5q+
Me-73	12q+
Me-96*	1q+
Me-102	9p+,22p+

* Also 1-3 d.m. in each metaphases.

Structural abnormalities

Structural rearrangements were seen in all abnormal karyotypes (Fig. 4). Prominent abnormalities were deletion and unbalanced translocations, which also lead to partial deletions of the chromosome arm involved. Inversions were seen a few times, particularly in rearrangements of chromosomes 1, 2 and 11; duplications and isochromosomes were unfrequently found.

Fourteen balanced translocations, all different, were identified in 11 cases. Chromosomes 3 and 4 were involved 4 times and chromosome 10, 3 times. Breakpoints were not recurrent but 3p and 10q were involved 3 times each (Table 3, Fig. 5). Chromosome amplification, as identified by HSR and dm were seen in only 5 cases (Table 4, Fig. 6).

No clear specific rearrangements or breakpoints were found but some chromosomal changes were clearly non-random: i.e. (partial) loss of 3p, 4, 6q, 9p and 22, while chromosomes 13, 16, 17 and 19 showed many "whole arm" rearrangements with breakpoints around the centromere (p11 or q11). For chromosome 13 e.g. unbalanced translocation onto the short arm (13p+ marker) was found in at least one third of the cases and numerical loss of 13 was often compensated by translocation of the whole long arm onto another chromosome. Structural abnormalities of chromosome 1 were very frequent, involving both arms and they were not always fully interpretable. Furthermore both chromosomes 1 were often rearranged, some markers were present in 2 or more copies and thus no clear picture emerged concerning specific breakpoint, and partial gain or loss of chromosome 1 material.

In contrast, chromosome 2 was seldomly modified, although deletion or rearrangement of the short arm did occur a few times and pericentric inversion was

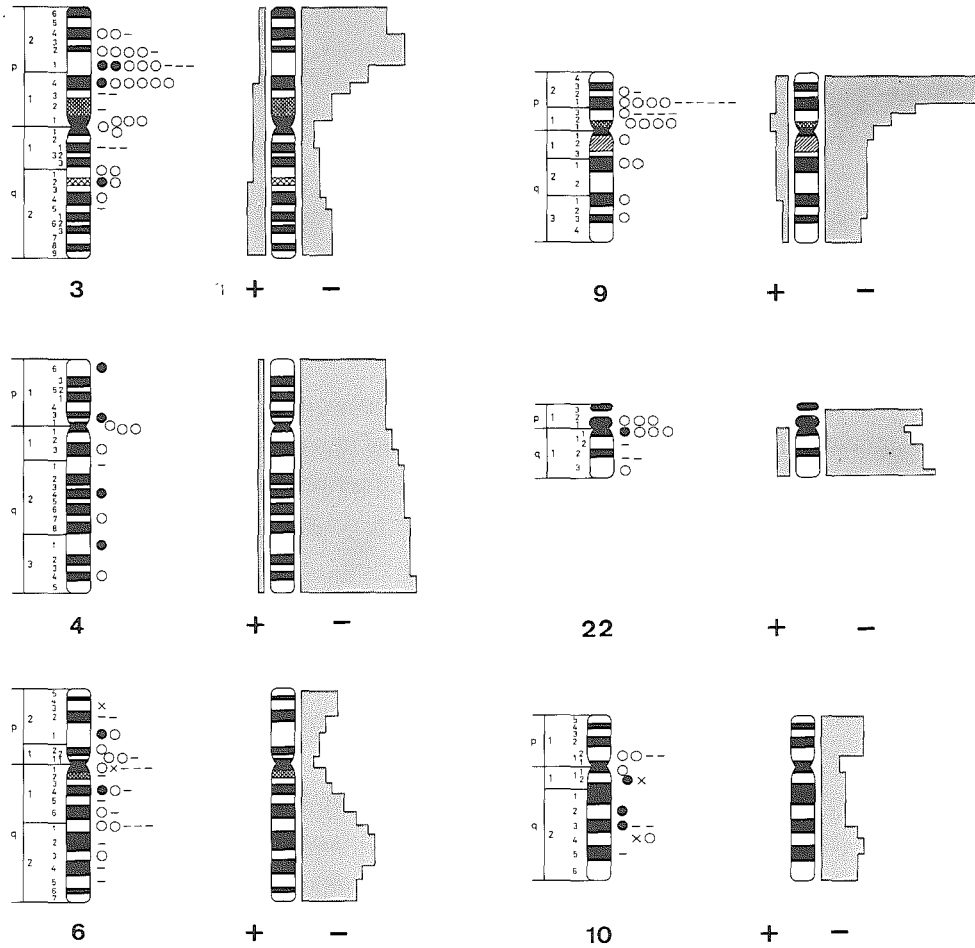

Figure 7. *Schematic representation of changes in chromosome 3, 4, 6, 9, 10 and 22. On the left the sites of breakpoints in structural rearrangements: ●: balanced translocation; O: unbalanced translocation; X: inversion; - deletion. On the right, the resulting gain (+) or loss (-) of chromosomal material.*

found in at least 4 instances.

The non-random changes of chromosomes 3, 4, 6, 9 and 22 are illustrated in detail in Fig. 7.

Abnormalities of chromosome 3 were seen in 27 (90%) of the 30 abnormal karyotypes: particularly frequent were deletions and unbalanced translocations of the short arm. The shortest region of overlap of these partial losses appeared to be around band 3p21. Three of the balanced translocations had also breakpoints on the short arm of chromosome 3 i.e. 3p14, 3p21 and 3p21. Loss of a complete chromosome 3 was seen in 2 patients in part of the cells, while additional marker chromosomes resulting in gain of the long arm were seen in 3 karyotypes: Me-9, Me-13 and Me-14.

Loss of the short arm of 9 was the most consistent feature of the hypodiploid and hypotetraploid karyotypes: only one case (Me-19) of 24 did not show alteration of chromosome 9 and in 3 cases (Me-41, Me-83 and Me-96) there was even apparent nullisomy for 9p. In contrast, among 6 hyperdiploid cases, one showed trisomy 9 (Me-62), 4 a normal pair of 9 and only one case a marker 9p+ with loss of the short arm distal to p21.

Loss of chromosome 4 was a non-random event in the hypodiploid and hypotetraploid karyotypes: i.e. in 13 cases monosomy 4 and in 7 cases unbalanced translocations resulting in (partial) loss of the long arm (6x) or short arm (1x). Gain of # 4 was seen only once (Me-13). Loss of chromosome 22 was also found in more than half of the cases, particularly in the hypodiploid and hypotetraploid categories.

Chromosome 6 is the site of many structural rearrangements that do not always lead to loss or gain of material; among these 2 balanced translocations (Me-50 and Me-83), a pericentric inversion (Me-84) and various markers in Me-13 and Me-72. Loss of the short arm was seen 6 times and of (part of) the long arm 14 times. Chromosome 10 was involved in 3 balanced translocations and one inversion. There is a small clustering of breakpoints between 10q22 and q24. Examples of stemline karyotype are given Figures 8-10.

Cytogenetics of control pleural effusions

Cytogenetic analyses of 14 non-malignant pleural effusions was also performed using the Direct method. 287 Metaphases were analyzed showing mostly a normal karyotype with 2% tetraploid cells, 8% of non-clonal random loss (among which 2% with -Y) and 2% non-clonal structural changes, half of these in a single case that had previously been irradiated. We also studied 208 metaphases from 10 metastatic effusions of various carcinoma. All showed a mixture of normal metaphases (n = 100) and cells with very abnormal karyotypes. The modal chromosome numbers were: <46

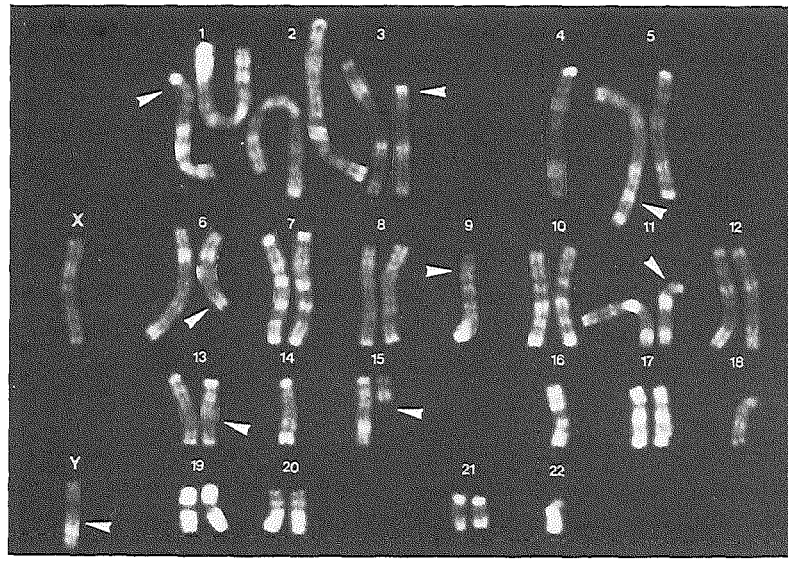

Figure 8. *Stemline karyotype of Me-26 with a hypodiploid karyotype, demonstrating the non-random loss of # 4, 9, 14 and 22, deletions of chromosomes 1p, 3p, 6q, 13q, rearrangement of 9 and two balanced translocations: t(5;11) and t(Y;15). The loss of 16 and 18 are incidental in this metaphase. R-bands with acridine orange.*

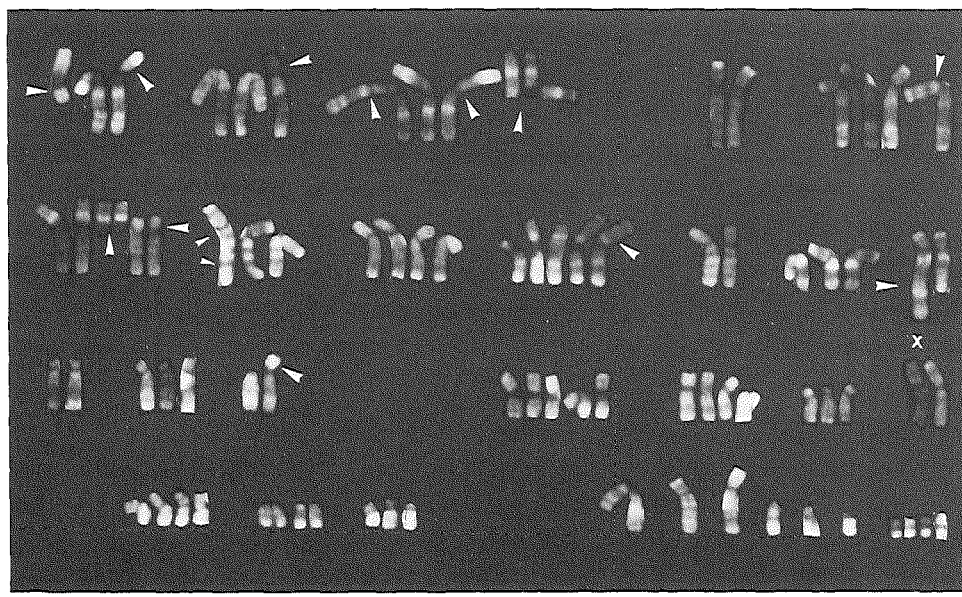

Figure 9. *Stemline karyotype of Me-14 with a hypotetraploid karyotype: there are two copies of most markers. The 2p- and 5p+ and some of the unidentified markers are changes unique to this cell. R-bands with acridine orange.*

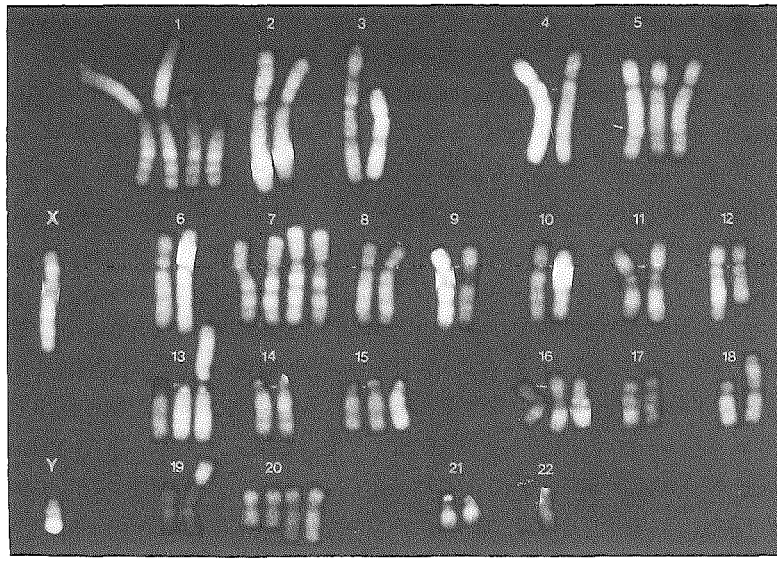

Figure 10. *Stemline karyotype of Me-95 with a hyperdiploid karyotype, demonstrating gain of 1q, 5, 7 and 20, a translocation (3;18) and various structural rearrangements. QFA banding technique.*

in 3 cases, 65-70 in 6 cases and 100-120 in one case. Metastic effusion of thyroid carcinoma, breast carcinoma and choriocarcinoma (one each) showed highly abnormal karyotype with multiple breaks, large abnormal markers, rings, d.m., HSR. In contrast, lung adenocarcinoma metastases showed multiple abnormalities that were not strikingly different from malignant mesothelioma: structural changes of chromsomes 1 and 3 were almost constantly present. However, the typical association pattern of abnormalities found in MM was usually missing and the modal chromosome number was often higher in lung adenocarcinoma. Unfortunately, at this stage of the study, a cytogenetic marker allowing a safe discrimination between lung adenocarcinoma and malignant mesothelioma was not found.

DISCUSSION

Cytogenetic analysis of 40 malignant mesotheliomas failed in one case, showed a normal karyotype in 9 cases and an abnormal karyotype with multiple and complex rearrangements in the 30 remaining cases. The latter were very heterogeneous and

an obvious primary abnormality was not detected: indeed a specific change common to all abnormal cases was not found and none of the karyotypes showed only a single or a few changes. Our analysis of the data showed two major types of chromosomal evolution: 1) in 24 out of 30 abnormal cases, chromosome loss and rearrangements resulted in a hypodiploid karyotype, that later on became hypotetraploid by duplication, endoreduplication or rarely by cell fusion (Me-84). This pattern of evolution showed non random loss of chromosomes 4 and 22, and structural changes leading to partial deletion of 3p and 9p. In the hypodiploid category rearrangement of the long arm of 11 and 12 were also frequent. Further clonal progression proceeded by random (?) loss of chromosomes towards a near triploid modal chromosome number (as seen in most reported cell lines) eventually followed by duplication towards a modal chromosome number of 120-125 (Me-25). This type of karyotypic evolution i.e. hypodiploidy to hypotetraploidy has also been observed in other tumors e.g. colon carcinoma (24). 2) In 6 of the 30 abnormal cases we observed an hyperdiploid chromosome number, with non-random gain of chromosomes 7, 5 and 20. Deletion of 3p was also often found but not monosomy 4 or deletion of 9p, while chromosome 22 was structurally rearranged in 3 cases. This second cytogenetic type could be relatively more frequent in other studies of MM, which could explain some of the discrepancies between our and other reported cases (11,14-16). In some of these cases gain of chromosome 7 appeared to be the first (maybe significant) sign of aneuploidy (case 16 in ref. 11 and case 1 in ref. 14). These hyperdiploid cases showed also a higher level of genetic instability giving rise to various, sometimes apparently unrelated, clones e.g. Me-50, Me-69 and Me-82 in this report and the case 1 reported by Stenman et al. (1986). The latter appears as an extreme example of this phenomenon. Structural abnormalities of chromosomes 1, 5, 6, 10, 13 and 17 were ubiquitous, although not present in all the cases. They involved various breakpoints resulting in variable unbalance of genetic material and thus they were considered as frequently occurring secondary changes.

Regarding the normal karyotype observed in 10 cases, our interpretation is that the metaphases analyzed were not of tumoral origin but that they were produced by reactive mesothelial proliferation, macrophages and other inflammatory cells always present in these exsudates and by contaminating fibroblasts and macrophages in cultures.

We found no correlation between cytogenetic category and clinical variables as age of onset, primary site, survival time and histological type of tumor. The fibromatous histological type represents about 10% of the tumors: 4/40 in this report, 3/30 in (11), 0/12 in (10) and 1/9 in (16); it is rare in male, but in female patients it is found in about half of the cases. The relative prevalence of fibromatous mesothelioma in older

women could indicate that exposure to asbestos is not the major etiological factor for that type of tumor and that other aggression could play a role, like previous irradiation therapy as reported (25).

Cytogenetically, these fibromatous MM appeared also heterogenous although our 4 cases (Me-20, Me-82, Me-88 and Me-96) shared some less frequent abnormalities like +5, +7p, +20 (3x each), but also more common changes: -4, -16, -22, structural abnormalities of # 1, # 14 and # 18 that were seen in at least 3 of the 4 cases.

Cytogenetic analysis of 62 MM, using banding techniques were previously reported: i.e. 11 cell lines (*in vitro* and xenografts) and 51 fresh tumor material (effusions and biopsies)(9-19). These studies also showed prominent heterogeneity, complex structural changes and absence of a common marker.

Furthermore, comparison of the reported cases between them and with our series fails to disclose a similar pattern of karyotypic changes. Tiainen et al. (11) e.g. agree with our study on non-random loss of 4, 22, and 9 p, but they emphasized the gain of # 7, 11 and 12 which we did not observe to such extend and Gibas et al. (10) found rearrangement of 9p in only 2 out of 9 cases while 9q was affected 5 times. Some of the variations between studies may be due to difference in the way of analysing the data, particularly concerning the numerical changes of tumors with high modal chromosome numbers. The other discrepancies are less easy to explain.

Previous cytotoxic therapy of patients before cytogenetic analysis and many passaging *in vitro* of cell lines will undoubtly induce secondary changes. But many cases, and all ours, were studied before treatment and thus the heterogeneity in structural changes is most probably genuine and related to genetic differences between patients, for instance, chromosomal fragile sites could be individually and geographically different. Differences in type and size of asbestos fibers used could also induce particular chromosomal aberrations.

Presently, chysotile (white asbestos) is the most widely used and less carcinogenic than amosite (brown asbestos), croccidolite (blue asbestos) and zeolite mined in Turkey. But, this has not always been the case and the long lag phase (25-40 years) between exposure and tumor development, and the movement of workers from South of Europe to Northern industrialized countries made it almost impossible to collect thrustworthy epidemiological data. All asbestos kinds are contaminated by other fibers. The remnants found in lungs and tumors are from large particles that are responsible for asbestosis, but the small thin (0.5 - 2.5 μm diameter, 10 - 80 μm long) acid resistant fibers that are thought to be responsible for tumoral transformation of mesodermal tissue are rarely found in tumors and even less in effusion cells. Asbestos related malignant mesothelioma did occur in isolates but unfortunately these tumors were not cytogenetically analyzed (26).

This extensive cytogenetic study of primary (untreated) malignant mesothelioma had two major aims: Firstly to see if a characteristic cytogenetic change could be found that would be specific for (subtypes of) MM and of help in diagnosis and prognosis as it is the case for other tumors (27) and secondly to investigate whether (specific) genetic abnormalities could provide a clue towards the mechanism of oncogenesis in these tumors. Regarding the first point, we did not find a consistent karyotypic change (see discussion above) nor a cytogenetic marker allowing safe discrimination between lung adenocarcinoma and malignant mesothelioma. The absence of a specific translocation or chromosomal breakpoint associated with MM makes it impossible to investigate concretely a mechanism of oncogene activation by specific chromosomal rearrangement like activation of c-*abl* in chronic myeloid leukemia with t(9;22)(28) or c-*myc* activation in lymphomas with chromosomal translocations involving 8q24 (29).

We observed a non-random pattern of chromosome losses i.e. of 9p and 3p as well as # 4 and # 22, particularly in the hypodiploid/hypotetraploid types. This suggest that in these tumors loss of suppressor genes located on these particular chromosomes are playing a role in the development of the cancer, although more studies are needed to establish this probability. In hyperdiploid karyotypes, loss or rearrangement of 3p was also seen, but it was not always accompanied by loss of an allele, which emphasize again the need to study these cases for loss of constitutional heterozygosity, at these particular loci, using polymorphic markers [30].

In another study we tested these tumors and cell lines derived from these tumors for expression of various oncogenes and an abnormally high expression of c-*sis* was consistently found [31].

This finding does not correlate with cytogenetic findings as c-*sis* maps on chromosome 22 and loss of # 22 is a non-random event in these tumors. The high expression of c-*sis* (B-chain of PDGF) is most probably related to the mesodermal nature of these tumors and the role played by c-sis enhanced expression in MM proliferation is under investigation.

In MM, two findings are consistant: 1) asbestos exposure and 2) complex and heterogeneous cytogenetic changes. It has been demonstrated *in vivo*, in animal experimentation and *in vitro* that asbestos fibers penetrate living cells, where they induce chromosomal breakages and rearrangements [32] and also that they stimulate chromosomal integration of DNA fragments in transfection assays [33]. Furthermore, for unknown reason, mesodermal tissue is 10 to 100x more sensitive to the action of asbestos fibers than bronchogenic epithelium. A scenario can be hypothesized where asbestos fibers penetrate mesodermal cells, where they induce multiple chromosomal breaks and rearrangements. Some chromosomes carrying fragile sites will be

preferentially involved and this may vary individually, geographically and with the type of fibers used. As a consequence benign mesodermal proliferation may be induced but also malignant mesothelioma, when specific unbalance, e.g. loss of particular suppressor genes has occurred. This scenario would account for the long lag phase and the high cytogenetic heterogeneity observed. In this hypothesis, multiple, random chromosomal changes precede the occurrence of "primary" specific (and presumably oncogenic) alterations which makes their identification particularly arduous. Such a scheme could be tested *in vitro* using cultures of normal bronchogenic epithelium and normal mesodermal cells to determine their sensitivity to and the genetic damage induced by various amounts of asbestos fibers of various types. This approaches was already followed by other investigators, but so far only limited results have been obtained [32.33].

ACKNOWLEDGMENTS

Supported by the Dutch Cancer Society. The authors express their gratitude to Professor Hilvering and all the Chest Physicians of Rotterdam and the province of Zeeland for referring their patients. We thank Prof. D. Bootsma and Prof. R. Benner for advice and support. We thank P. Zondervan for pathological diagnosis early in the study. The expertise and help of Dr. H.T. Planteydt and the other members of the Dutch mesothelioma panel for the pathological diagnosis of these tumors was always appreciated. The technical assistance of E.M.E. Smit and M. Delahaye and the secretarial assistance of R. Boucke are gratefully acknowledged.

REFERENCES

1. McDonald JC, McDonald AD (1977): Epidemiology of mesothelioma from estimated incidence. Prev Med 6: 426-446.
2. Lilienfeld DE, Mandel JS, Coin P, Schuman CM (1988): Projection of asbestos related disease in the United States, 1985 - 2009. I. Cancer. Br J Ind Med 45: 283-291.
3. Whitaker D, Shilkin KB (1984): Diagnosis of pleural malignant mesothelioma in life - a practical approach. J Pathol 143: 147-175.
4. Yunis JJ (1983): The chromosomal basis of human neoplasia. Science 221: 227-236.
5. Turc-Carel C, Philip I, Berger MP, Philip T, Lenoir GM (1984): Chromosome study of Ewing's sarcoma (ES) cell lines. Consistency of a reciprocal translocation t(11;22)(q24;q12). Cancer Genet Cytogenet 12: 1-19.
6. Mertens F, Johansson B, Mandahl N, Heim S, Bennet K, Rydholm A, Willen H, Mitelman F (1987): Clonal chromosome abnormalities in two liposarcomas. Cancer Genet Cytogenet 28: 137-144.
7. Turleau C, de Grouchy J, Chavin-Colin F, Junioen C, Seger J (1985): Cytogenetic forms of retinoblastoma: Their incidence in a survey of 66 patients. Cancer Genet Cytogenet 15: 321-334.
8. Whang-Peng J, Kao-Shan CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, Minna JD (1982): Specific chromosome defect associated with human small cell lung cancer: Deletion 3p(14-23). Science 215: 181-182.
9. Mark J (1978): Monosomy 14, monosomy 22 and 13q-. Three chromosomal abnormalities observed in cells of two malignant mesotheliomas studied by banding techniques. Acta Cytol 22: 398-401.
10. Gibas Z, Li FP, Antman KH, Bernal S, Stahel R, Sandberg AA (1986): Chromosome changes in malignant mesothelioma. Cancer Genet Cytogenet 20: 191-201.
11. Tiainen M, Tammilehto L, Mattson K, Knuutila S (1988): Non random chromosomal abnormalities in malignant pleural mesothelioma. Cancer Genet Cytogenet 33: 251-274.
12. Ayraud N (1975): Identification par dénaturation thermique ménagée des anomalies chromosomiques observées dans six tumeurs metastiques humaines. Biomedicine 23: 423-430.
13. Wake N, Slocum HK, Rustum YM, Matsui S, Sandberg AA (1981): Chromosomes and causation of human cancer and leukemia. XLIV. A method for chromosome analysis of solid tumors. Cancer Genet Cytogenet 3: 1-10.

14. Stenman G, Olofsson K, Monsson T, Hagmar B, Mark J (1986): Chromosomes and chromosomal evolution in human mesotheliomas as reflected in sequential analyses of two cases. Hereditas 105: 233-239.
15. Bello JM, Rey JA, Avilés MJ, Arevalo M, Benitez J (1987): Cytogenetic findings in an effusion secondary from pleural mesothelioma. Cancer Genet Cytogenet 29: 75-79.
16. Popescu NC, Chahinian AP, DiPaolo JA (1988): Nonrandom chromosome alterations in human malignant mesothelioma. Cancer Res 48: 142-147.
17. Shanfang W, Enzhong W, Jianzhong S, Huixuan X, Zhenqiong L, Cifu S, Changwen X, Ruiming C, Xiuzhen Y, Dehou Z and Zhenghong Y (1985): Establishment and characterization of human malignant pleural mesothelioma Cell Line SMC-1. Scientia Sinica (Series B) XXVIII 281-286.
18. Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden A (1987): Characterization of a human malignant mesothelioma cell line (H-MESO-1): A biphasic solid and ascitic tumor model. Cancer Res. 47: 3199-3205.
19. Hsu S-M, Hsu P-L, Zhao X, Kao-Shan CS, Whang-Peng J (1988): Establishment of human mesothelioma cell lines (MS-1, -2) and production of a monoclonal antibody (Anti-MS) with diagnostic and therapeutic potential. Cancer Res. 48: 5228-5236.
20. Versnel MA, Bouts MJ, Hoogsteden HC, Van der Kwast ThH, Delahaye M, Hagemeijer A (1989): Establishment of human malignant mesothelioma cell lines. Int J Cancer 44, 256-260.
21. Versnel MA, Hoogsteden HC, Hagemeijer A, Bouts MJ, Van der Kwast ThH, Delahaye M, Schaart G, Ramaekers FCS (1989): Characterization of three human malignant mesothelioma cell lines. Cancer Genet Cytogenet, in press.
22. Trent J, Crickard K, Gibas Z, Goodacre A, Pathak S, Sandberg AA, Thompson F, Whang-Peng J, Wolman S (1986): Methodologic advances in the cytogenetic analysis of human solid tumors. Cancer Genet Cytogenet 19: 57-66.
23. Van der Kwast ThH, Versnel MA, Delahaye M, De Jong A, Zondervan PE, Hoogsteden H (1988): Expression of epithelial membrane antigen on malignant mesothelioma cells. Acta Cytol. 32: 169-174.
24. Muleris M, Salmon RJ, Dutrillaux B (1988): Existence of two distinct processes of chromosomal evolution in near-diploid colorectal tumors. Cancer Genet Cytogenet 32: 43-50.
25. Brenner J, Sordillo PP, Magill GB, Golbey RB (1982): Malignant mesothelioma of the pleura. Review of 123 patients. Cancer 49: 2431-2435.
26. Driscoll RJ, Mulligan WJ, Schultz D, Candelaria A (1988): Malignant mesothelioma. A cluster in a native american pueblo. New Eng J Med. 318: 1437-1438.
27. Gibas Z, Prout GR, Conolly JG, Pontes JE, Sandberg AA (1984): Nonrandom chromosomal changes in transitional cell carcinoma of the bladder. Cancer Res. 44: 1257-1264.
28. De Klein A (1987): Oncogene activation by chromosomal rearrangement in chronic myelocytic leukemia. Mutation Res 186: 161-172.
29. Croce CM (1987): Role of chromosome translocations in human neoplasia. Cell 49: 155-156.
30. Van den Berg A, Kok K, Vos A, Oosterhuis JW, Li FP, Hagemeijer A, Buys CHCM (1989): Are chromosome 3 deletions a consistent abnormality in mesotheliomas? Cancer Genet Cytogenet 38: 203.
31. Versnel MA, Hagemeijer A, Bouts MJ, Van der Kwast ThH, Hoogsteden HC (1988): Expression of c-sis (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene 2: 601-605.
32. Sincock A, Seabright M (1975): Induction of chromosome changes in Chinese hamster cells by exposure to asbestos fibres. Nature 257: 56-58.
33. Lechner JF, Tokiwa T, LaVeck M, Benedict WF, Banks-Schlegel S, Yeager H, Banerjee A, Harris CC (1985): Asbestos-associated chromosomal changes in human mesothelial cells. Proc Natl Acad Sci USA 82: 3884-3888.
34. Appel JD, Fasy TM, Kohtz DS, Kohtz JD, Johnson EM (1988) Asbestos fibers mediate transformation of monkey cells by exogenous plasmid DNA. Proc Natl Acad Sci USA 85: 7670-7674.

APPENDIX

DETAILED CULTURE AND CYTOGENETIC DATA OF 40 CASES OF MALIGNANT MESOTHELIOMA

Patient Nr. (1)	Cytogen. Class (2)	Material & Technique (3)	Number of Cells			Stemline Karyotypes (4)
			Tot.	Norm. Clonal	Abn.	
ME-3	II	P1, DM	30	0	30	85(81-86),XXYY,+del(1)(p31),+del(1)(p31),+del(1)
(ME-8)		P2, DM	48	35	13	(q23),+del(1)(q23),+i(2q),+i(2q),+5,+5,+5(+5),
		cult.	21	21	0	+6,(+6),+7(+7),+der(8)t(3;8)(p14;q22),+der(8)
						t(3;8),+10,+11(+11),+12,+12,+13,+14,(+14),+15,
						+15,+16,+16,+18,(+18),+19,+19,(+19),+20,+20,
						(+20)+21,+22,+22.
Me-9	I	P, DM	17	8	9	44-46,XY,1q-(1pter→q11::?),3p-(3qter→p14::?),
						t(4;10)(q24;q23),(-6),7q+(7pter→q21::HSR?::3q)
						-8,9p-,mar.inv(11),13q+,14q+,-19,-20,+mar
						(20p;17q), + 2 to 4 markers.
						87-90, same duplicated (25%).
Me-10	II	P, DM	81	72	9	81-83,XXY,del(1)(p21p23),del(1)(p21p23),+der(1)
		line	10	4	6	t(1;8)(p21;q22),+der(1)t(1;8),+inv(2)(q22p11,
						+inv(2)(q22p11),+der(3)t(3;15)(p22;q21),+del(4)
						(q21),+del(5)(q13q34),del(6)(q11q21),+del(6)
						(q11q21),+del(6)(q11q21),+7q+[7pter→q11::7q22→
						q11::1p21→pter],+7q+,8q+,(8pter→q24::?13q),+8q+
						(idem),+del(9)(p21),+del(9)(p21),+del(10)
						(q23q25),+inv(11)(q14q24),+12,+12,+14,+14,-15,
						-15,+16,+16,+17,+18,+der(20),t(15;20)(q21;p13),
						+der(20)t(15;20),+21q+,+21q+,+22,+ring,+mar C,
						+2 mar G.
						167: 1 cell, duplication of all markers.
Me-13	III	P, DM	15	3	12	61,XXY,+2 mar 1p-(6p?::1q),+1p+(1qter→p35::6q21
		cult.	18	18	0	→ter or 13q),+2,+3p+(3qter→p14::5q),+4,-6,+mar
						6p-q+(6p1→q21::8q),+7,+7,-8,+11,+11q-,13p+
						(13q::5q)+mar inv13p+,+15,+16,17p+(22q?::17q)
						+20,+20p+,22p+(22q::?8p).

Me-14	II	P,	DM	22	18	4	75(72-78),XX,-Y,-Y,der(1)t(1;?)(p11;?),der 1p-(idem),+t(1p19q),+t(1p19q),
			cult.	18	0	18	+2,del(3)(p22p24),del(3)(p22p24),+der(3)
			line	13	0	13	t(1;3)(q11;p21),+der(3)t(1;3),+der(3)t(3;?)
							(q11;?),+i(3p),+5,+5,+,+der(6)t(6;?)(p11;?),
							+6p- idem,+del(6)(q11),+del(6)(q11),+mar 7
							(7pter→cen::?7q32-q11::19p or q)+8,(+8),+del
							(9)(p21),+9p+(dup 9 p21p22 or HSR),+9p+ idem,
							+11,(+11),12q+ [? ins (12;3)(q23;q12q28)],
							(+12q+,idem) +14,15p+,+16,+mar 16,+mar 16,
							+17,+17,(+18),-19,-19,+i(21q),mar 22,
							+ 7 unidentified markers.
Me-19	I	P,	DM	31	16	3(+12)*	45,XX,1p+,inv.(1p)q-,-2,2p-,-4,5q+,6q-,-8,mar 8q-?
			cult.				31 31011q-,der(12)t(12;?)(p11;?),+13q+,-22,+R,+2 mar.
							*Abnormal cells, same markers, not fully analysable.
Me-20	I	P,	DM	12	8	3	42-43,XY,(-Y),del(1)q31,t(3;10)(q22;q22),-4,del(6)
			cult.	9	6	3	(q22),-7,9p-,10q-,14q+,del(16)(q21q23),-18,-22,
							(+mar).
							86, same duplicated markers (one cell).
Me-21	N	P,	DM	11	11	0	46,XY/92,XXYY (one cell).
			cult.	10	10	0	
Me-25	II	P,	DM	31	13	18	67(65-69), inv(X)(p22q12),+inv(X),Y,+Y,1p-
(Me-7)			cult.	24	24	0	(1qter→p11::Sat)(3-4 copies), +2 copies[del(1)
		B,	line	10	0	10	(q12)inv(1)(p36p13)],der(2)t(2;?)(p12;?),(+2),
							+del(3)(p21),+del(3)(p21),+del(5)(q21q34),
							+del(5),+6,(+6),+8(+8),del(9)(p21),mar(9q::19p),
							+10,(+10),+11,(+11),+mar inv.(11)(p11q14),-13,
							+17,inv(18)(p11q12),inv(18),(-19),der(20)
							t(20;21)(p11;q11)-21,-21,+22q+,+22q+,+mar
							(17q- or 19q+), +mar 16, +mar 16.
							135 (127-140) same markers, in 15 cells from
							pleural effusion.

Me-26	I	P,	DM	32	16	16	42,X,t(Y;15)(q12;q14 or q15), del(1)(p21p35),
(Me-37)		A,	DM	46	14	32	mar 2 [2qter→q11::9p13→q31::?], del(3)(p21),
			cult.	16	5	11	-4, t(5;11)(q35;p13),del(6)(q16q24),-9, mar
							9p-(9qter→q21::18 like),del(13)(q22q32),
							-14,-22.
							84, same with duplication of markers,
							in 4 pleural effusion cells.
							N.B. in ascites, del(1)(p21p35) is replaced
							by ring.
Me-27	I	P,	DM	47	11	36	44,XY,del(1)(p12p21),del(3)(p14p22),-4,-6,
							mar(8)[8q::10p),-9,-10,der(11)t(8;11)
							(q22;q23),der(13)t(6;13)(p11;q22),14p+
							(14q::4p),der(17)t(17;?)(q24;?),-22,
							+3 different E size markers.
							One tetraploid and one octoploid cell.
Me-31	N	P,	DM	34	34	0	46,XY/92,XXYY (4 cells).
Me-41	II	P,	DM	2	1	1	72(70-75),XXYY,+inv del(1)(?1q22→cen::1p31
			cult.	8	4	4	→p35::1q24→qter),+inv del(1),del(3)(q13),
			line	12	0	12	del(3)(q13),+del(3)(p12)+mar(3)(14q- or
							13q-::cen::?::3q),+mar 3, inv(5)(p15q11),
							inv(5),+der(5)t(5;6?)(p15;q15?),+7,+7,
							t(8;17)(p11;q11),t(8;17),del(9)(p21),del(9)
							(p21),+9p+,+9p+,+10,+11,+11,+del(12)(p11p12),
							+del(12),-13-13,+del(14)(q23q31),t(15;22)
							(q21;q11),t(15;22),+15,+16,+17,+17,+18,
							+20q+,+20q+,+21,+21,+i(21q).
Me-45	failure	P,	DM				Hemorrhagic fluid, no analysable metaphase.
			cult.				
Me-48	II	A+B*	cult.	59	11	48	71-75,XXYY,+der(1)t(1;?)(p33;?2p or 10q),+2,
			lines	120	0	120	+der(2)t(2;9)(p11;p11),+der(2)t(2;9;?)
							(2pter→p11::9 cen::?)+3,+3,t(4;10)(p11;q11),
		(*various metastatic sites)					t(4;10),+del(5)(q11q22),+5q+(HSR),+6(+6),
							+7q-,+7q-,+7,+8,(+8),+9(+9),+10,+mar(11p:9p),
							+11,+12,i(13q),13q+(1p),+17,+19,+del(20)(q12),
							+21,+21p+ or i(21q),+22,+mar.

Me-50	III	P	cult.	38	11	27	48,XY,+inv(2)(q13q32),+del(3)(p14p22),t(4;6;13)(p16;q14;q13),+7,inv(10)(q11q24),-18,20q+,-22,+mar (11%). 51,Xq+,Y,1q-(1pter→cen::2p21pter),+2p-[del(2)p12p16)inv(2)(q13q32],+del(3)(p14p22),inv.(5)(p11q12),+7,9p+(9qter→p21::1q12→qter)+12p+[12qter→q13::1q21→pter(11%) or 12qter→q13::1q21→p22(89%)],13q+(13pter→q33::16p),del(13)(q22q32),del(14)(q23q31),15q+(15pter→q24::?)del(16)(p11),16q+(16pter→q22::?),+mar (89%).
Me-51	N	P,	DM	30	30	0	46,XY/46,XY,inv(2)(p16q21) in 2 cells.
Me-56	II	P,	DM	12	4	8	83(78-86)X,YY,+del(1)(p33),+del(1)(p33),+2,+2,t(3;12)(p21;q23),t(3;12),+3,+3,der(4)t(4;?)(q27;?),+der(4),del(5)(q23q34),5p+(18q::5q),+5p+,+6q-(6p::16p),+6q-,+6q-,+7,+7,t(8;14)(q21;q23),t(8;14),+8q-(del(8)(q21) or der 8p20p),+8q-,+9p+[9qter→p11::?22q11→qter),+11,+11,+12,+12;+del(13)(q21)+del(13)(q21),+14,+14,+17,+17,+18,+18,+19,+21,+21,+ring,+mar, + fragments.
			cult.	26	22	4	
Me-57	N	P,	DM	30	30	0	46,XY
Me-61	III	P,	cult.	8 4(-Y)	4	0	49,XY,1p-q+(1pter→p33::1p22→q42::13q11→qter),3p-(3qter→p11::?),+5p-q-(del(5)(p12p14) and del(q11q13)),+del(8)(q12q21),-10,11p+(int. dup),-13,mar 14(?inv q24q12),17p+,+mar 19 (?inv p+q-),20q-,21p+,+mar G (?18q-). 96-97, duplicated stemline (20-25%). 45,X,-Y (50% of presumably normal cells).
		B1	cult.	8	8		
		B2	cult.	13	4(-Y)	9	
Me-62 (Me-69)	III	P,	DM	8	7	1	51-53,XY,+del(3)(p21),-4(50%),del(6)(p22),+8,+9,+12,+15,+16,+21,non-clonal t,i.e.t(4;10)(p15;q21)(one cell) and t(3;8)(p;p)(one cell).
			cult.	16	13	3	

Me-64	II	P,	DM	5	0	5	75-81,Xq+,Y,+del(1)(q12),+der(1)t(1;6)(q11;p11),
			cult.	10	0	10	+1,+2,+inv(2)(p23q12?),der(3)t(3;12)(q24 or 25;q11),+der(3)t(3;12),+4,+del(5)(q21q33), +5,+6,+7,+7,i(8q),+i(9q),+10p+(21q?),+del(11)(q21),+12,+12q+,i(13q),+der(13)t(7;13)(p11;p11),+14p+(?21q),+14,+15q+(tand dic.t (15q;13q),+16,+16,+17p+(18q::17q),+17,+18,+19, +20,+20,+21p+(18?),+22p+(7p or 11p),+22p+, (+22)(+22).
							70-71 after culture: variable unstable karyotype with additional, complex rearranged markers.
Me-67	N	P,	cult.	16	16	0	46,XY
Me-72	I	P,	DM	12	7	5	42,XY,del(1)(p32),inv(1)(q23q43),del(3)(p13p24),
		B	cult.	7	0	7	-4,del(6)(p22), del(6)(q16),+der(6)t(6;17)
			line	28	0	28	(q23;q11),der(8)t(8;16?)(p11;p11?),del(9)(p12), -13,der(13)t(4;13)(q11;p11),-14,der(16)t(16; 17)(p11;q11)-17,-17,-22,+mar (22q-? or 17p?).
Me-73	I	P,	DM	11	0	11	43-44, mar(X),Y,-1,del(1)(q42),-2,der(3)t(3;5)
			cult.	14	11	3	(q22;q13),-4,4p+,del(5)(p14),5q-,del(6) (q12q24),del(7)(q35),(-7),8q+,-9,10p+(2q?), del(11)(q21),11q+[11pter→q24::20 like], 12q+[12pter→q22::?HSR],13p+(2p?),15q+,15q-,mar 18 or -18,-19,-21,-22,+ fragments, + ring, + 2 to 4 markers G and E size (14%).
							74-86, same markers (57%).
							147-155, same markers (29%).
Me-77	N	P1,	DM	26	0	26	46,XY
(Me-43)		P1,	DM	16	0	16	
(Me-54)		P3,	cult.	21	5	16	45,X,-Y (5 cells)/92,XXYY (4 cells).
Me-78	N	P,	DM	14	0	14	46,XY
(Me-76)			cult.	13	0	13	

Me-82	III	B,	cult.	11	0	11	48-50,XX,+5,+mar(7p::22q),der(14)t(8;14)(q13;p11) der(14)t(8;14),+20,+21,with extensive inter-cellular variations, and variable structural changes of chromosomes 1p, 1q, 12p, 22 etc.
							94-98, same markers (50%).
Me-83	I	P,	DM	11	8	3	42,XY,1p-,1q-p+(inv 1p11q31,del 1q),2p+(2qter
			cult.	8	0	8	→p21::?12q),del(2)(q31q34,der(4)t(4;22) q11;q11),+der(4)t(4;15)(q13;q21),del(5) (q22q34),t(6;17)(p21;p12),+der(6)t(6;17),-6, -7,-8,del(9)(p12),del(9)(p13),-10,11p+ (11qter→p15::?),der(12)t(7;12)(q22;q23)-14, -15,18q+[18pter→q22::11q21→pter),20p+,-22, +mar E (in pleural effusion cells).
							75-85,XXY, duplication of most markers, in addition no 2p+,-3,2x10q+(10pter→q23::? 12q),1x 14q-, 2x 18q-,-Y,+ring,+mar C (inv 12?) (in cultured cells).
Me-84	I	P,	DM	16	0	16	38,X,-Y,1p-(1q::13q),-3,-4,inv(6)(p23q11 or q12),
			cult.	8	1	7	-9,der(10)t(Y;10)(q12;q24 or 25),-11,13p+ (13q::3q),-13,-16,der(17)t(9;17)(q12;p13) -18,der(22)t(11;22)(q13.1;q13.3).
							76, same duplication of stemline (20%).
		B	cult.	15	10	5	81-98,XXY, one copy of markers only = fusion of normal diploid with abnormal hypodiploid: 5 cells from biopsy culture.
		line		20	0	20	38, same stemline with t(6p+;11p-),+der(11)t (11;22),-der(22)t(11;22).
							76, same duplication of stemline (5%).
Me-85	I	P,	DM	16	4	12	43-46,XY,del(1)(p11p31),1q+,inv(2)(p22q11),
			cult.	20	20	0	del(3)(p13),3q+,der(5)t(5;7)(q12;q11), del(6)(q16), del(6)(q21 or q22), del 7p15, -7,-9, dup(12)(q13q22),del(12)(q12q15), 13p+(?21),-14,14p+,15p+(?7p),de;(16)(q22), +mar C (67%)

							75-87 same with duplication of most markers excepted: not present: 1p-,1q+,der(5),15p+ new markers in one or 2 copies: 1q-(1pter→q24::?), 2p-,del(5)(q12q34), 10p+, mar 15(? inv q13q25),17p+, del(18)(q22q23) (33%).
Me-86	N	P	cult.	30	30	0	46,XY
Me-88	II	P,	DM	11	9	2	73,XXX, +inv.(2)(p24?q32),+del(3)(p22p24) +del(3),+5,+5,+5,+del(6)(q16?q24?),+7, +8p+,-9,10p-,+12p+(13q),13p+,+15,-16, +17,+17,+17q+,+17q+,+18q-,+20,+20, +20,+21,+21,+large mar,+2 small mar.
	II						
Me-94	N	P,	DM	10	10	0	46,XY
Me-95	III	P,	DM	16	8	8	54-58,XY,t(3;18)(p14;p11),+5,+7,+7,+8 or
(Me-97)		B1,	cult.	30	30*	0	8p-(19p?::8q),+11,del(12)(q21q23),13p+,
		B2, 2	cult.	13	4	9	-15,+2 mar(15)(inv del),+16,der(19),
		line		11	0	11	t(11;19)(q13;p12?),+20q+,+20q+,der(22) t(1;22)(q11;q11). + markers (ring or C or D).
							*45,X,-Y in 24/30 cells from B1.
Me-96	II	B1,	cult.	23	0	23	72-78,XXYY,+del(1)(q11),+2,+3,+4,+5,+5,+5, +6q-(6p::11p?),+6q-,+7q-(7p::16q?),+7q-, +8p+,+8p+,del(9)(p21),-9,(+11),+12,(-13), +14,15q+,15q+,+17,+inv(18)(p11q21),+inv (18),+19,+20,+20,+20,+21,+21,+22q-,+22q-, +1-2 large marker (1q::HSR),+dm,+variable markers eg. 1p-(5p::1q)14p+,22p+,19p-q-, etc.).In general unstable karyotype, chromosome number varying between 59 and 80 and in 3 cells 122 to 140 with duplicated stemline.
		line		17	0	17	Same stemline with additional markers: 2p- (1 copy), 17p+ (2 copies), 4 copies # 4 and 2 copies # 5.
Me-100	I	P,	DM	4	0	4	46,XX,dup(1)(p12p22),del(3)(p21p24) (1 cell)
			cult.	17	2	15	45,XX,dup(1)(p12p22),del(3)(p21p24),t(3;4) p(21;q34),del(10)(p11),del(17)(p12),-22 (41%).

							89,XXXX, same stemline, tetraploid (1 cell). 41 same markers and in addition -X,-1,-9,-12,-15,+mar 1p-q+(? inv dup 1q) der(10)t(10;12)(p15;q12) (47%).
Me-102	I	P,	DM	21	4	17	41-42,XY,-1,del(3)(p21),-4,der(5)t(5;19?)(q33; p11),del(7)(q21 or q22), der(8)t(8;19?) (q23;q11), 9p+(4q or HSR),ins(11)(q22;p11 p12),del(12)(q12q23),der(13),t(1;13) (p11;q11),-14,-15,-16,17p+(?dup),18?,-19, 20?,i(21q),-21,22p+(HSR),+ring(?15),+3 discrete small markers.
			cult.	14	4	10	80-82, same with stemline duplication (24%).
Me-104 (Me-113)	II	P,	DM	15	3	12	90,XXY,+del(1)(q22),+del(1)(q22),+2,+2,+3q-(3 pter→q13::?),+3q-,+der(4)t(3;4)(q21; q35),+der(4)t(3;4),+6,t(7+14)(q35;q12), t(7;14),+7,+7,+7,+7,+del(7)(q22),+del (7)(q22),+mar(8),+mar(8),del(9)(p21), +del(9)(q13q33),+del(9)(q13q33),+11,+11, +12,+12,+der(13),t(13;16)(p13;q11),+der (13)t(13;16),+14,+14,+15,+16q-(16pter→ cen::?),+16q-,+16q-,+17,+17,+18,+18,+19, +19,+20,+20,+21,+21,+mar.
			cult.	20	5	15	175-189, same duplicated stemline (11%).
Me-105	I	P,	cult.	45	30	15	45,XY,dup(1)(q32→q43),del(1)(p12p31)(q31), der(2),t(2;15)(p11;q15),-3,-5,?ins(6;3) (q16;q13q25),der(6),t(5;6)(q12;q21),-7, der(7),t(7;14)(q31?;q21?),der(9)t(7;9) (q22;p21),inv(11)(q13p14,+del(11)(q13), +inv(11)p+(?13q or 6q), del(12)(q12),12q+, -13,-14,-14,15s+,mar(15),del(16)(q23), der(19),t(12;19)(q21;q13),+21,+mar i(5p), +mar i(17p).

1. Patient no. consistently used in text and table, in parentheses no. of additional sampling occurring at another date. 2. N = normal karyotype, no clonal abnormalities found, I: hypodiploid, II: hypotetraploid and III: hyperdiploid modal chromosome number. For clinical and pathological data see Tables 2 A, B, C and D. 3. P = pleural fluid, A: ascites fluid, B: biopsy, P1, P2, refer to different sampling of same patient. DM = Direct method, cult.: culture, line: established cell line from the same patient's material. 4. In parentheses clonal changes observed in only part of the cells. Numerical changes are given relatively to the normal diploid karyotype (46,XY or 46,XX), also in the hypotetraploid stemline, thus different from the analyses done in Fig. 3 (see text).

CHAPTER 6

HUMAN MALIGNANT MESOTHELIOMA CELL LINES

6.1 Establishment of human malignant mesothelioma cell lines

6.2 Characterization of three human malignant mesothelioma cell lines

CHAPTER 6.1

ESTABLISHMENT OF HUMAN MALIGNANT MESOTHELIOMA CELL LINES

Marjan A. Versnel, Monique J. Bouts, Henk C. Hoogsteden[1], Theodorus H. van der Kwast[2], Mick Delahaye[2] and Anne Hagemeijer

Department of Cell Biology, Immunology and Genetics, [1]Department of Pulmonary Medicine and [2]Department of Pathology, Erasmus University and Academic Hospital Dijkzigt, Rotterdam, The Netherlands

International Journal of Cancer 44, 256-260, 1989.

SUMMARY

Seventeen human malignant mesothelioma cell lines were isolated from 61 samples (46 effusions, 9 biopsies and 6 tumors obtained at autopsy) collected from patients with a confirmed malignant mesothelioma. The method used is given in detail. Cytogenetic analysis of growing cultures is the best indicator to determine whether the observed proliferation concerns malignant or normal mesothelial cells. The addition of epidermal growth factor (EGF) and hydrocortisone (HC) or EGF alone, to the culture medium increases the chances of successful isolation of a malignant mesothelioma cell line.

INTRODUCTION

Human malignant mesothelioma is a neoplasm originating from mesodermally derived tissues of the coelomic cavities. Epidemiological studies have established that exposure to asbestos fibers is the primary cause of mesothelioma in the industrial world (Wagner et al., 1960). The latent period for mesothelioma ranges from 15 to over 40 years and the incidence of malignant mesothelioma is increasing. At initial presentation most malignant mesothelioma patients have a pleural or peritoneal effusion. A reliable diagnosis of malignant mesothelioma on effusions by routine cytology is limited by the difficulty of distinguishing malignant mesothelioma cells from benign mesothelial cells and adenocarcinoma cells (Whitaker and Shilkin, 1984). Even with the use of additional techniques such as immunocytochemistry, many malignant mesotheliomas are still not diagnosed until autopsy.

Viable tumor tissues of malignant mesotheliomas for cell biological and cytogenetic investigations are difficult to obtain. Several groups have reported the successful transplantation of human malignant mesotheliomas into nude mice (Chahinian et al., 1980; Reale et al., 1987), but so far the production of *in vitro* growing human malignant mesothelioma cell lines has been extremely difficult. Only a few permanent human malignant mesothelioma cell lines have been produced (Ohnuma et al., 1979; Behbehani et al., 1982; Tibbets et al., 1984; Shanfang et al., 1985; Anderson et al., 1986), and detailed data concerning the isolation procedure have not been published.

Over the last few years we have established a panel of 17 human malignant mesothelioma cell lines. The characterization of 3 of these has been described (Versnel et al., 1989). We now report in detail the procedures and methods used for the isolation of these mesothelioma cell lines from pleural effusions and solid tumor mate-

rial, as well as the methods of culturing normal mesothelial cells from non-cancerous effusions. These cell lines are extremely useful tools for investigation of the properties of malignant mesothelioma cells and the development and application of new diagnostic methods.

MATERIAL AND METHODS

Processing of patient material

Pleural effusions

Pleural fluids were collected under sterile conditions in 50-ml tubes with 5,000 I.U. heparin (Organon, Oss, The Netherlands) and 5 ml Ham's F10 medium (Gibco, Paisley, UK), the latter being supplemented with 15% fetal calf serum (FCS) and antibiotics. After collection, the material was immediately transported to the laboratory. The cells were harvested by centrifugation (10 min, 200 g) and the cells from 1-2 tubes were plated in a 75-cm^2 culture flask (Costar, Cambridge, MA). After overnight incubation at 37°C the medium containing the non-adherent cells, mostly lymphocytes and erythrocytes, was removed and replaced by fresh medium.

Biopsy and autopsy material

Autopsy should be performed within 4 hr of death in order to obtain viable samples. At autopsy, different sets of instruments were used for incision of the skin and removal of the anterior part of the chest wall. Tumor material was obtained preferably from lateral parts of the pleural cavity which had not been previously touched by instruments. Material from different parts of the primary tumor and metastases was transferred to 50-ml tubes with 5 ml F10 medium containing 15% FCS and antibiotics. In the culture room the material was placed in a Petri dish (60 x 15 mm, Costar, Cambridge, MA) and fat, necrotic tissue and lung tissue were removed. For each sample new instruments were used. Pieces of approximately 2 mm^3 were obtained by mincing with scissors and incubated (37°C, 5% CO_2 in air) overnight in 1 ml F10 medium with 15% FCS and 8 mg/ml collagenase (125 U/mg, Cooper, San Diego, CA). The digested material was resuspended with a pipette and incubated for another 20 min (37°C). After transfer to a tube and centrifugation (5 min, 200 g) the cells were plated in a Petri dish with 2 ml F10 medium containing 15% FCS.

Culture conditions

Cells were cultured in F10 medium with 15% fetal calf serum (FCS), 0.1 mg/ml streptomycin-sulfate (Biochrom KG, Berlin, FRG) and 10^2 UI/ml penicillin G sodium (Gist-Brocades, Delft, The Netherlands). If enough patient material was available, the cells were plated on 3 different media: F10 medium with 15% FCS, F10 medium with 15% FCS and 10 ng/ml epidermal growth factor (EGF; Collaborative Research, Lexington, MA) and F10 medium with 15% FCS, 10 ng/ml EGF and 0.4 μg hydrocortisone (HC; Pharma Chemie, Haarlem, The Netherlands).

Subcloning was performed by limiting dilution in 96-well tissue culture plates (Costar).

Cytogenetics

For cytogenetic studies of cells in pleural effusions, the fluid was collected in 50 ml tubes containing 5 ml F10 medium with antibiotics, heparin and 1-2 μg colcemid/tube (Gibco). After 30-60 min the cells were centrifuged and metaphases harvested according to standard cytogenetic procedures. This method is referred to as the "direct method".

Metaphase cells from the cultures were obtained by shaking culture flasks with exponentially growing cells and collecting the supernatant. After addition of colcemid for 15 min the metaphases were harvested. RFA-, QFQ- and GTG-banding techniques were used and the karyotype was established according to the ISCN (1985).

Detection of intermediate filaments

Gel electrophoresis, immunoblotting and immunofluorescence were performed according to Broers et al. (1986). Antibodies used were the chain-specific antibodies RCK102, RCK105, RV202, RGE53, RCK106 and LP2K (Ramaekers et al., 1983; 1987; Lane et al., 1985).

RESULTS

Patient material

Of the 117 samples collected from patients with suspected malignant mesothelioma, 61 were derived from 51 patients whose malignant mesothelioma was diagnosed on the basis of cytology, histology and/or electron microscopy (Table I). The diagnosis of malignant mesothelioma was never based on cytology alone. To enable a definite diagnosis to be made, the availability of material processed for histology and/or electron microscopy was considered essential. The samples not

TABLE I

Incidence of malignant mesothelioma for collected patient material and isolated cell lines

Collected patient material		Diagnosis of mesothelioma	Successful isolation of a malignant mesothelioma cell line
Effusions	85	46	10
Biopsy	21	9	2
Autopsy	11	6	2*

* Five cell lines were isolated from 2 autopsies.

diagnostic for malignant mesothelioma were effusions from adenocarcinoma metastases (5), from other malignancies (3), or from effusions reactive to lung and pleural infections (11); in the case of 29 samples the diagnosis was not determined. From the 61 samples derived from 51 malignant mesothelioma patients (46 effusions, 9 biopsies and 6 autopsies) 17 human malignant mesothelioma cell lines were isolated.

Method of establishment of malignant mesothelioma cell lines

From all collected samples cultures were started as described in "Material and Methods". At the time of appearance of small groups of growing cells, these were subcultured by trypsinization in the same flask or dish and, when confluence was reached, transferred to a new flask. Cells were stored in liquid nitrogen and cytogenetic analysis was performed. When a small percentage of chromosomally abnormal cells was found, immediate subcloning was performed to prevent overgrowth of malignant cells by normal mesothelial cells, so that a fully abnormal cell line could be isolated. Growing subclones were screened again for cytogenetic aberrations. Cultures showing no growth or only cytogenetically normal cells were kept for at least 2 months as we had observed in a few instances that the malignant mesothelioma cells started growing after 2 months.

Malignant mesothelioma cell lines from autopsy and biopsy material

Four malignant mesothelioma cell lines were isolated from tumor obtained at autopsy of 2 malignant mesothelioma patients, and 2 others from pleural biopsies (Table II). These cell lines were isolated on F10 medium with 15% FCS and no other supplementation.

Isolation of cell lines from autopsy material was often hampered by infections

from contiguous lung tissue. In fact, metastases outside the thoracic cavity were a better source of material, as demonstrated by the 4 cell lines Mero-48a, b, c and d obtained from a single patient (Table II). These 4 cell lines have similar growth patterns, morphology and cytogenetics (Table III).

Biopsies were found to be a poor source of material for cultures: they are usually very small and sometimes had been obtained blindly with an Abrams needle. Usually a number of very small biopsies are taken, and pathological diagnosis cannot be performed on the same sample that is used for culture.

Malignant mesothelioma cell lines from effusions

Ten malignant mesothelioma cell lines were isolated from 7 effusions under varying culture conditions (Table IV). We found no direct relationship between the percentage of tumor cells (varying from morphologically not detectable to 60%) and the success-rate of establishment, probably because very cellular fluids often contain many non-viable cells. Hemorrhagic fluid seemed unfavorable for the isolation of cell lines.

Connell and Rheinwald (1983) reported that the addition of EGF and HC promotes the growth of normal mesothelial cells. Initially we did not add EGF and HC to the culture medium used for the establishment of mesothelioma cell lines. We found, however, that the growth of the malignant mesothelioma cell lines Mero-14 (from effusion) and Mero-25 (from autopsy material) was inhibited by EGF and HC, while

TABLE II

Origin of isolated human malignant mesothelioma cell lines from solid tumor material

Cell line	Patient material	Histology of tumor
Mero-25	Autopsy pleura	Biphasic
Mero-48a	Autopsy pleura	Biphasic
Mero-48b	Omentum	Epithelial
Mero-48c	Liver	Epithelial
Mero-48d	Pericard	Biphasic
Mero-72	Biopsy pleura	Biphasic
Mero-96	Biopsy pleura	Fibrous

Table III

Cytogenetic data of the malignant mesothelioma cell lines

Cell lines	Number of chromosomes (range)	Consistent numerical change [1]	Number of structural abnormalities in chromosomes number:										
			1	3	5	6	9	11	12	13	17	22	other
Mero-14[2]	72-75	-4,-10,-13,-18,-19,-20,-21	2	3		1	1		1			1	2
Mero-25[2]	127-140	-4,-7,-9,-12,-13,-14,-15,-18,-19	2	1	1		1		1			1	2
Mero-41[2]	68-75	-2,-4,-6,-13	1	2	2		2		1		1	1	4
Mero-48a,b,c,d	71-75	-10,-14,-16	1	1	2	1		1		2	2		7
Mero-72	41-43	-4,-13,-14,-17,-22	2	1		3	1			1		1	2
Mero-82	48-50/94-98	+5,+20,+21,-X	2						1			1	2
Mero-83	42/75-85	-7,-8,-10,-14,-15,-22	3		1	1	1	1	1		1		
Mero-84a,b,c	38/76	-4,-9,-11,-13,-16,-18	1	1		1	1	1		2	1	1	2
Mero-91	42-43/84-86	-2,-4,-22				1	1		1				3
Mero-95a,b	54-58	+5,+7,+7,+11,+16	1	1					1	1		1	5
Mero-96	55-78	+2,+5,-7,+8,-9,+14,+20,+21	1	1		1					1		3

1) Reference karyotype diploid/tetraploid respectively - unidentified markers and rearranged chromosomes are not included, 2) Full description of karyotype in Versnel (1989).

TABLE IV

Characterization of mesothelioma cell lines isolated from pleural effusions

Malignant mesothelioma cell lines isolated	% of morphologically detected tumor cells cells in the effusion	% of cytogenetically abnormal cells in the effusion	Growth of cytogenetically abnormal cells in culture medium[2] supplemented with:		
			[1]No additions	EGF	EGF + HC
Mero-14	<5	18	+	ND[3]	ND
Mero-41	50	40	-	-	+
Mero-64	<5	100	-	-	+
Mero-82	<5	ND	-	ND	+
Mero-83	60	10	-	+	-
Mero-84a,b,c	30	100	+	+	+
Mero-95a,b	50	40	+	ND	+

[1]detected with the direct method, [2]F10 + 15% FCS, [3]ND: not done.

addition of EGF only slightly enhanced their proliferation (Versnel et al., 1989). After this observation we started to culture malignant mesothelioma cells not only in F10 medium with 15% FCS but also in the same medium supplemented with EGF or with both EGF and HC. Seven cultures of pleural effusions resulted in the successful isolation of malignant mesothelioma cell lines. Five cell lines were isolated from medium supplemented with EGF and HC, 2 from medium supplemented with EGF only and 3 from unsupplemented medium (Table III). The addition of EGF and HC or EGF alone to the medium used for the isolation of malignant mesothelioma cell lines did increase the overall efficiency of establishment. After their establishment, none of these cell lines required EGF and/or HC for growth. Cell lines isolated independently under different culture conditions but originating from the same effusion show similar cytogenetic features.

Normal mesothelial cell cultures

Non-cancerous effusions, mostly from patients who had heart failure were used for the establishment of normal mesothelial cell cultures. These effusions were cultured on medium supplemented with EGF and HC according to Connell and Rheinwald (1983). The normal mesothelial cultures had a limited lifespan of approximately 15 passages and proliferating cells had a normal karyogram. Cytological analysis was consistent with the mesothelial morphology of the cells and expression of cytokeratins and vimentin was found. Normal mesothelial cells were also often seen in pleural effusions of malignant mesothelioma patients. These cells quickly became adapted to *in vitro* conditions and tended to overgrow the malignant mesothelioma cells, which

emphasizes again the need for early subcloning in the procedure of isolation of malignant mesothelioma cell lines.

Characteristics of the malignant mesothelioma cell lines

After establishment, there was no intrinsic difference between lines derived from effusion cells or from solid tumor material. Each cell line has its own *in vitro* properties but they share features characteristic of their (malignant) mesothelial origin.

1. Cell morphology. The malignant mesothelioma cell lines showed a spindle shaped or epithelial appearance with numerous nucleoli. Multinucleate cells and mitotic figures did frequently occur in all malignant mesothelioma cell lines. These characteristics were absent in cultured normal mesothelial cells.

   All malignant mesothelioma cell lines expressed the epithelial membrane antigen (EMA) which was not present on cultured normal mesothelial cells (Van der Kwast et al., 1988). Figure 1 shows the morphology of the malignant mesothelioma cell lines Mero-25 and Mero-48b. Ultrastructural studies of Mero-14, Mero-25 and Mero-41 showed that these cell lines have characteristics consistent with malignant mesothelioma (Versnel et al., 1989).
2. Growth properties. The doubling time of the malignant mesothelioma cell lines was variable. For some cell lines the doubling time was approximately 24 hr (e.g. Mero-82) and for others (e.g. Mero-25) it was 48 hr. The malignant mesothelioma cell lines did not undergo senescence, which occurred with cultured normal mesothelial cells at approximately passage 15. The characteristics of the cell lines were not changed by freezing and thawing using an established procedure for conservation of viable cells (10% DMSO, liquid N_2).
3. Intermediate filament expression. All malignant mesothelioma cell lines expressed several cytokeratins and vimentin as detected with specific mAbs against these filaments. Three cell lines (Mero- 14, Mero-25 and Mero-41) were studied for intermediate filament expression by immunoblotting. These results confirmed the presence, detected by immunofluorescence of vimentin and the cytokeratins 18, 8, 7 and 19. These findings are consistent, although not exclusive for the mesothelial origin of the cell lines.
4. Cytogenetics. All cell lines exhibited an abnormal karyotypes (Fig. 2, Table III). At the beginning, only cell lines with a high modal chromosome number could be isolated. Later on, hypodiploid and hyperdiploid lines were also successfully established. Each cell line has its own specific karyotype with specific markers which are similar to those found in fresh tumor cells of this particular patient, and which persist after subcloning. Tetraploidization is responsible for the high or biphasic modal chromosome number as most of the markers are found in duplicate. Intercellular variation exists and is mainly due to irregular loss of chromosomes, particularly of normal chromosomes with a high copy number. An example of hypodiploid karyotype is given in Figure 2. Analyses of the data did not reveal a particular translocation or abnormality specific for this type of tumor.

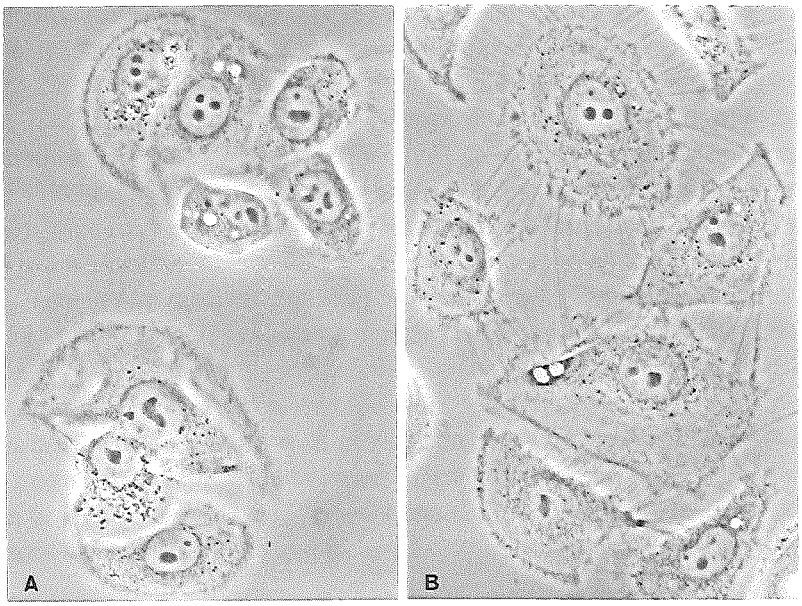

Figure 1. *Phase contrast morphology of the malignant mesothelioma cell lines Mero-25 (a) and Mero-48b (b) in tissue culture.*

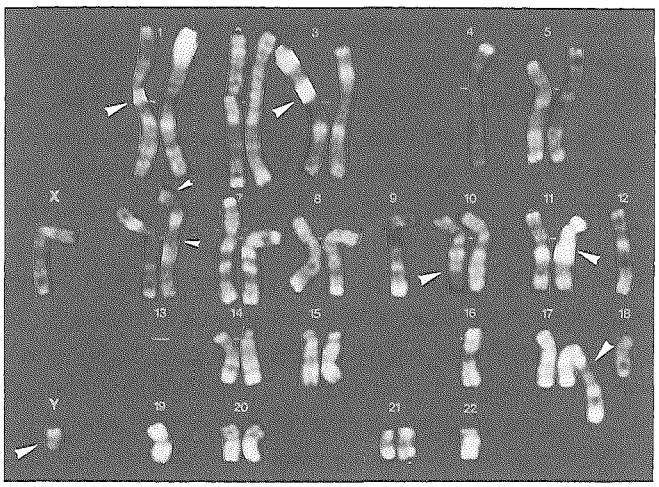

Figure 2. *Hypodiploid karyotype from Mero-84: arrows indicate structural rearrangements. 37, X, t(Y;10)(q12; q24 or 25), 1p-(13q::1q), -3, -4, inv(6), -9, -11, -12, 13p+(13q::3q), -13, -16, der(17) t(9;17)(q12;p13), -18, -19, der(22) t(11;22) (q13.1;q13.3). Loss of 12 and 19 is incidental in this particular cell. The large majority of Mero-84 metaphases showed also loss of the der(Y). The der(13) has been aligned to chromosome 11 because of partial homology.*

Preferential loss of some chromosomes (e.g. 4) and frequent involvement of some chromosomes (e.g. 1, 3, etc.) in marker formation is indicated in Table III.

5. Tumorigenicity. The malignant mesothelioma cell lines Mero-14, Mero-41 and Mero-48a, b, c were repeatedly injected into 8- to 12-weeks-old BALB/c nu/nu female mice (Bomholtgard, Rij, Denmark) subcutaneously or intraperitoneally, but never produced tumors. S.c. injection of 10^8 cells of Mero-25 produced a tumor on one occasion (size over 1 cm^3). Histologically, this tumor exhibited the same features as the original tumor cells of the patient Me-25 and it was possible to grow these cells *in vitro*. Further passage through nude mice was not attempted. Growth in semi-solid medium (methylcellulose 3%) of Mero-14 and Mero-25 was attempted a few times and was never successful.
6. Oncogene expression. The cell lines were tested in Northern blot analysis for expression of oncogenes including c-*myc*, c-H-*ras*, Ki-*ras*, c-*fms*, but no significant differences from normal mesothelial cells were found. Interestingly, elevated expression of the c-*sis* (PDGF-B) and PDGF A-chain genes compared to normal mesothelial cells has been found in 10 malignant mesothelioma cell lines studied to date (Versnel et al., 1988).

DISCUSSION

So far, 17 human malignant mesothelioma cell lines resulted from the processing of 61 samples from patients suffering from malignant mesothelioma. The addition of EGF and HC or EGF alone to the culture medium appears responsible for the elevated success rate since these growth factors were employed. However, their effect on individual samples is unpredictable. When Mero-83 was established, no proliferation of malignant mesothelioma cells was detected in medium with EGF and HC, while in the culture medium with EGF only a malignant mesothelioma cell line was isolated. On the other hand, for Mero-41 and Mero-64 our observations were different: cultures in medium with EGF and HC resulted in malignant mesothelioma cell lines while in the cultures with EGF alone no malignant growth could be detected. This is why no supplementation was used when biopsies were cultured. The size of the samples was too small so that one type of culture had to be chosen. We know that medium with EGF and HC also stimulates growth of normal mesothelial cells.

Cytogenetic analysis of growing cultures is the best indicator of whether the observed proliferation can be attributed to the malignant mesothelioma cells or to normal mesothelial cells. After subcloning, each malignant mesothelioma cell line was 100% cytogenetically abnormal and had a quite stable karyotype. The cytogenetic aberrations in the malignant mesothelioma cell lines are consistent with the karyotype of the tumor cells present in the original patient material. The various malignant mesothelioma cell lines obtained from pleural effusions and solid tumor tissue proliferate *in vitro* without showing signs of senescence.

ACKNOWLEDGEMENTS

We are grateful to Prof. Dr. R. Benner for his continuous support, and Dr. J.J.M. van Dongen, Dr. H.J. Adriaansen, Ms. E.M.E. Smit, Ms. E. Schoenmaker, Ms. M. Moret and Ms. I.L.M. Wolvers-Tettero for their advice and/or technical assistance. We thank Dr. F.C.S. Ramaekers (University Hospital of Nijmegen, The Netherlands) for kindly providing antibodies against intermediate filaments. We also thank the lung physicians in the area of Rotterdam and Vlissingen for providing the patient material and Ms. J. de Goey-van Dooren for typing the manuscript. This study was supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

REFERENCES

Anderson, T.M., Shin, W.-Y., Benedict, W.F., Holmes, E.C. and Saxton, R.E., Establishment of a human mesothelioma cell line. Proc. Amer. Ass. Cancer Res., 27,31 (1986).

Behbehani, A.M., Hunter, W.J., Chapman, A.L. and Lin, F., Studies of a human mesothelioma. Hum. Path. 13,862-866 (1982).

Broers, J.L.V., Carney, D.N., Klein Rot, M., Schaart, G., Lane, E.B., Vooijs, G.P. and Ramaekers, F.C.S., Intermediate filament proteins in classic and variant types of small cell lung carcinoma cell lines: a biochemical and immunochemical analysis using a panel of monoclonal and polyclonal antibodies. J. Cell Sci., 83,37-60 (1986).

Chahinian, P., Beranek, J.T., Suzuki, Y., Bekesi, J.G., Wisniewski, L., Selikoff, I. and Holland, J.F., Transplantation of human malignant mesothelioma into nude mice. Cancer Res., 40,181-185 1980).

Connell, N.D. and Rheinwald, J.G., Regulation of the cytoskeleton in mesothelial cells: reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell, 34,245-253 (1983).

Lane, E.B., Bártek, J., Purkis, P.E. and Leigh, I.M., Keratin antigens in differentiating skin. Ann. N. Y. Acad. Sci., 455,241-258 (1985).

Ohnuma, T., Wolman, S.R., Daum, S.M. and Holland, J.F., Establishment and characterization of a human mesothelioma cell line. Proc. Amer. Ass. Cancer Res., 20,162 (1979).

Ramaekers, F., F., Huysmans, A., Moesker, O., Kant, A., Jap, P., Herman, C. and Vooijs, P., Monoclonal antibody to keratin filaments, specific for glandular epithelia and their tumors. Use in surgical pathology. Lab. Invest., 49,353-361 (1983).

Ramaekers, F., Huysmans, A., Schaart, G., Moesker, O. and Vooijs, P. Tissue distribution of keratin 7 as monitored by a monoclonal antibody. Exp. Cell Res., 170,235-249 (1987).

Reale, F.R., Griffin, T.W., Compton, J.M., Graham, S., Townes, P.L. and Bogden, A., Characterization of a human malignant mesothelioma cell line (H-MESO-1). A biphasic solid and ascitic tumor model. Cancer Res., 47,3199- 3205 (1987).

Shanfang, W., Enzhong, W., Jianzhong, S., Huixuan, X., Zhenqiong, L., Cifu, S., Changwen, X., Ruiming, C., Xiuzhen, Y., Dehou, Z. and Zhenhong, Y., Establishment and characterization of human malignant pleural mesothelioma cell line SMC-1. Sci. Sin., 28,281-288 (1985).

Tibbetts, L.M., Chu, M.Y., Spremulli, E.N., Leduc, E.H. and Calabresi, P., Loss and restoration of tumorigenicity in a human mesothelioma cell line. Proc. Amer. Ass. Cancer Res., 25,45 (1984).

Van der Kwast, Th.H., Versnel, M.A., Delahaye, M., de Jong, A., Zondervan, P.E. and Hoogsteden, H. Expression of epithelial membrane antigen on malignant mesothelioma cells. Acta Cytol., 32,169-174 (1988).

Versnel, M.A., Hagemeijer, A., Bouts, M.J., van der Kwast, Th.H. and Hoogsteden, H.C. Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene, 2,601-605 (1988).

Versnel, M.A., Hoogsteden, H.C., Hagemeijer, A., Bouts, M.J., Van der Kwast, Th.H., Delahaye, M., Van Schaart, G. and Ramaekers, F.C.S. Characterization of three human malignant mesothelioma cell lines. Cancer Genet. Cytogenet., in press (1989).

Wagner, J.C., Sleggs, C.A. and Marchand, P., Diffuse pleural mesothelioma land asbestos exposure in the North Western Cape Province. Brit. J. Indust. Med., 17,260-271 (1960).

Whitaker, D. and Shilkin, K.B., Diagnosis of pleural malignant mesothelioma in life - a practical approach. J. Path., 143,147-175 (1984).

CHAPTER 6.2

CHARACTERIZATION OF THREE HUMAN MALIGNANT MESOTHELIOMA CELL LINES

Marjan A. Versnel, Henk C. Hoogsteden[1], Anne Hagemeijer, Monique J. Bouts, Theo H. van der Kwast[2], Mick Delahaye[2], Gert Schaart[3] and Frans C.S. Ramaekers[3]

Department of Cell Biology, Immunology and Genetics, [1]Department of Pulmonary Medicine and [2]Department of Pathology, Erasmus University and Academic Hospital Dijkzigt, Rotterdam and [3]Department of Pathology, University Hospital of Nijmegen, The Netherlands

Cancer Genetics and Cytogenetics, in press.

ABSTRACT

Three human malignant mesothelioma cell lines, designated Mero-14, Mero-25 and Mero-41, have been isolated from effusions and from autopsy material of confirmed cases of malignant mesothelioma. Light and electron microscopy, cytogenetics, growth requirements, and intermediate filament expression of these cell lines were studied and, where possible, compared with the original tumor material of the patient. Cytologic and ultrastructural morphology was consistent with the mesothelial nature of the cells. All cell lines displayed a hyperdiploid karyotype similar to that of the tumor cells obtained directly from the patient. All three malignant mesothelioma cell lines had marker chromosomes 1, 3, 9 and 22, as well as other markers that were occasionally present in these cell lines and in other malignant mesotheliomas studied. Growth kinetic studies in medium supplemented with epidermal growth factor (EGF) showed increased proliferation and a decreased proliferation in medium supplemented with hydrocortisone (HC) or EGF plus HC. The three malignant mesothelioma cell lines were positive for the cytokeratins 7, 8, 18, and 19 based on immunofluorescence and immunoblotting tests with chain specific monoclonal antibodies. The characteristics of these cell lines support the assumption that Mero-14, Mero-25 and Mero-41 are derived from malignant mesotheliomas and have retained their original character.

INTRODUCTION

Human malignant mesotheliomas are rare tumors that occur most frequently in the pleura and less frequently in the pericardium and peritoneum. A strong relationship has been found between asbestos exposure and the development of a mesothelioma (1). Despite better controlled use of asbestos, the incidence of mesothelioma is still increasing, because of the long lag time between asbestos exposure and the appearance of the tumor.

The first symptom of malignant mesothelioma is usually a pleural effusion. Cytologic diagnosis is difficult as there are no consistent morphologic criteria for differentiation between benign and malignant mesothelial cells on the one hand and adenocarcinoma cells on the other (2). Additional techniques, such as immunocytochemistry, electron microscopy, morphometry, and chromosomal analysis have proved to be useful but are still not sufficient to establish the diagnosis of malignant mesothelioma with certainty in all cases (3-6).

Biochemical analyses of the cytoskeletal composition of mesothelioma cells have suggested the usefulness of intermediate filament typing. Cytokeratin 5 seems

to be a valuable marker for discriminating between malignant mesotheliomas and adenocarcinomas (7). Coexpression of vimentin and cytokeratins often occurs in malignant mesotheliomas and has also been proposed to be discriminative between mesotheliomas and adenocarcinomas (8-10). However, some of the latter cancers may occasionally show simultaneous expression of both cytokeratins and vimentin (11-14). Specific tumor cell lines are invaluable tools to gather more insight into the cellular and molecular biologic characteristics of malignant mesotheliomas. We report here on three continuously growing malignant mesothelioma cell lines that were established from patient material. Light and electron microscopy, cytogenetics, growth requirements and intermediate filament expression patterns of these cell lines have been studied and, where possible, compared to the original patient material.

MATERIALS AND METHODS

Patient material

Malignant mesothelioma cell lines were derived from pleural effusion of the patients Me-14 and Me-41 and from autopsy tumor material of patient Me-25. All three patients were men over the age of 60, with a history of asbestos exposure. All patients presented with dyspnea, weight loss, and a large pleural effusion on chest x-ray. There were no signs of other malignancies. All patients died within 1 year after the first symptoms.

Isolation of cell lines and growth conditions

Mero-14 and Mero-41 were isolated from the pleural effusion of patients Me-14 and Me-41, respectively. Mero-25 was isolated from autopsy material of patient Me-25. The effusion cells were collected in 50 ml tubes with 5000 IU heparin (Organon, Oss, Holland) and 5 ml Ham's F10 medium (Gibco, Paisley, United Kingdom) supplemented with 15% fetal calf serum (FCS), glutamine, and antibiotics. The cells were plated at high density and after 24 hours the medium containing non-adherent cells was replaced with fresh medium. The pleural fluid of patient Me-41 was also cultured in F10 medium supplemented with 10 ng/ml epidermal growth factor (EGF; Collaborative Research Inc., Lexington, MA) or 10 ng EGF and 0.4 μg/ml Hydrocortisone (HC).

When a continuously growing culture was established, cells were routinely subcultured two or three times a week by trypsinization. These three cell lines were the first three we isolated from a panel of seventeen human malignant mesothelioma cell lines (15). The investigations reported here were done after stable establishment of the cell lines between 3 and 6 months after sampling of material. The lines have

been frozen and thawed a number of times and have been subcloned later on. Cytogenetic analysis and intermediate filament expression were studied again 1 year later and the findings were consistent with earlier studies.

Cytology and transmission electron microscopy

For diagnosis, routine cytology was applied as described previously (2). For transmission electron microscopy, the sediments of pleural effusions were fixed in phosphate-buffered 4% paraformaldehyde (pH 7.2) and 1% glutaraldehyde, postfixed in 1% (w/v) OsO4 in 0.1 M phosphate buffer (pH 7.2) for 12 hours at 4°C, rinsed in the same buffer, acetone dehydrated, and Epon embedded. Cell lines cultured on Melinex plastic (I.C.I., Rotterdam, the Netherlands) were similarly prepared for routine transmission electron microscopy.

Cytogenetics

For cytogenetic studies of cells in pleural fluid, the effusion was collected in 50-ml tubes containing heparin and 1-2 μg colcemid per tube (Gibco, Paisley, United Kingdom). After 30-60 minutes metaphases were harvested following standard cytogenetic procedures.

For cytogenetic studies of cell lines, metaphase cells were obtained by shaking the culture flasks containing exponentially growing cell cultures. The supernatant was collected in centrifugation tubes and Colcemid was added for 15 minutes. The metaphases were then harvested as in the direct method. RFA-, QFQ- and GTG-banding techniques were used, and the karyotype was established according to the ISCN (1985).

Growth kinetics

Before the growth kinetic assays were performed, the cells were cultured for at least 10 days in F10 medium with 15% FCS and EGF, EGF plus HC, or HC only. Cells were cultured sixfold (4000 cells/culture) in the appropriate media in a 96 well tissue culture plate. At 18 hours before harvesting ^{3}H-thymidine (0.5 μCi/culture) was added and the samples were harvested on days 2, 3, 4, 5, and 6, respectively.

Immuno(cyto)chemistry of intermediate filament proteins

The intermediate filament protein expression patterns of the three cell lines were assayed by indirect immunofluorescence and Western blotting. Monoclonal antibodies and antisera that recognize various subsets of cytokeratins, vimentin, desmin, and controls were used. The specificity of the antibodies is given in Table 1, together with references to their preparation and characterization (16-20). The

numerical designation of the cytokeratins refers to the catalog of Moll et al. (21). The indirect immunofluorescence technique was performed as described previously (16).

Gel electrophoresis and immunoblotting assays

Cells were harvested from 75 cm^2 culture flasks, spun down using a MSE Minor centrifuge at 1200 g for 5 minutes, washed once with PBS and stored at - 20°C until use. Gel electrophoresis and immunoblotting were done essentially as described by Broers et al. (20).

TABLE 1

Intermediate filament protein (IFP) expression by cell lines Mero-14, Mero-25 and Mero-41 as detected by indirect immunofluorescence, using specific antibodies

Antibody	IFP recognized	Mero-14	Mero-25	Mero-41	References
pKer	Several cytokeratins	+	+	+	(16)
RCK102	Cytokeratins 5 and 8	+	+	+	(18)
RGE53	Cytokeratin 18	+	+	+	(17)
RCK106	Cytokeratin 18	+	+	+	(17)
RCK105	Cytokeratin 7	+	+	+	(18)
LP2K	Cytokeratin 19	+(5% of the cells)	+	+	(19)
RKSE60	Cytokeratin 10	-	-	-	(12)
pVim	Vimentin	+	+	+	(12)
RV202	Vimentin	+	+	+	(18)
RD301	Desmin	-	-	-	(20)
FITC conjugated rabbit anti-mouse-IgG	-	-	-	-	
FITC conjugated goat anti-rabbit-IgG	-	-	-	-	

RESULTS

Isolation of the cell lines

Two continuously growing cell lines called Mero-14 and Mero-25 were isolated in F10 medium with 15% FCS, from the effusion and autopsy material of patients Me-14 and Me-25, respectively. The pleural effusion of patient Me- 41 was cultured in F10 medium with 15% FCS, but also in F10 medium with supplementation of EGF plus HC or EGF only. In this case, a continuously growing cell line (Mero-41) was only obtained with F10 medium supplemented with EGF plus HC.

Histologic, cytologic, and ultrastructural characteristics of patient material and cell lines

Diagnosis of malignant mesothelioma was established by cytology, histology and/or electron microscopy. Clinical findings supported this diagnosis in the three investigated patients.

Histology revealed that patient Me-14 had an epithelial-type malignant mesothelioma. At the autopsy of patient Me-25, the pleura and the pericard were found to be diffusely infiltrated by cells with a spindle-shaped morphology as well as by strands of cells with an epithelial appearance (biphasic pattern). Transmission electron microscopy also revealed ultrastructural features characteristic of malignant mesothelioma, that is the tumor cells showed long slender villi, glycogen granules, and extensive intermediate-sized filament arrays.

The highly cellular pleural effusion derived from patient Me-41 contained several morulae of tumor cells with a collagenous core. Tumor cells expressed the epithelial membrane antigen (EMA), which is considered a marker for malignancy in serous effusions (15). Transmission electron microscopy showed the same ultrastructural features characteristic of the mesothelial nature of the tumor cells as described above.

Cell line Mero-14 consisted of spindle shaped cells with polymorphic and hyperchromatic nuclei, with prominent and atypical nucleoli. Ultrastructurally the cells displayed pseudopodiae, but slender villi were not observed. The cytoplasm contained some disperse glycogen granules, numerous polysomes, some Golgi membranes and several fat droplets. An extensive perinuclear intermediate filament network was present in addition to a terminal web of intermediate filaments.

Cell line Mero-25 displayed large polymorphic nuclei with a coarse chromatin pattern. Ultrastructurally, the cells with an epithelial appearance formed several slender villi projecting from the surface. Some tight junctions were seen at contiguous areas. The cytoplasm contained small numbers of disperse glycogen granules and numerous polysomes in addition to several Golgi membranes and a few fat droplets. Both dense

TABLE 2

Cytogenetic data of the mesothelioma cell lines Mero-14, Mero-25 and Mero-41

Patient cell line	Number of cells studied	Modal chromosome nr. (range)	Number of copies of normal chromosomes																						
			1,	2,	3,	4,	5,	6,	7,	8,	9,	10,	11,	12,	13,	14,	15,	16,	17,	18,	19,	20,	21,	22,	X,Y
Mero-14	13	75(72- 78)	0	3	0-1	2	4	2	2	3	2	2	3-4	1	2	3	2	3	4	2	0	1	2	1	20-1
Mero-25	15	135(127-140)	0	2-3	2-3	3-4	3-4	6-8	3-4	6-8	0	6-8	6-8	3-4	2-3	3-4	3-4	3-4	4-6	0	3	1-2	0	3-4	02-3
Mero-41	12	72(68- 75)	2-3	2	0	2	0	2	4	0	0	2-3	3-4	2	0	2-3	1-2	2-3	2	2-3	3-4	2	3-6	0-2	22

	Types of markers and number of copies in ( )	unidentified markers
Mero-14	1p-(2) 1q-(2) 3p+(2) i(3p)(2) 3p-(2) 6p-(2) Mar(7)(1) 9p+(2) 12q+(2) Mar16(2) Mar22(1)	7
Mero-25	inv(1)q-(3-4), 1p-(6-8), 2p+(2), 3p-(4), 5q-(3-4), 9p-(3), Mar(9q::19p)(3), inv(18)(3-4), 20p+(1-2) 22q+(3-4), inv(X)(2-3)	4
Mero-41	inv del (1)(2), 3p-(1), 3q-(2), Mar3(2), inv5(2), 5p+(1), 8p-(2-3), 9p-(2), 9p+(2), 12p-(2), 14q-(1) 15q-(1), 17p+(2), 20q+(2), i(21q)(1), 22q+(2)	1

and fine bundles of perinuclear intermediate filaments were present.

Cell line Mero-41 consisted of epithelial-like cells with large polymorphic nuclei. Occasionally, prominent nucleoli were found within the nucleus. Ultrastructurally, the cells showed a few villous structures and the cytoplasm contained several fat droplets and numerous polysomes, but only a few Golgi membranes. Both dense and fine bundles of intermediate filaments were found. Multinucleate cells occurred in all cell lines.

Cytogenetic characteristics

Metaphases obtained directly from effusions showed a mosaicism consisting of cells with a normal karyotype as well as highly abnormal cells with high numbers of chromosomes and a number of structural abnormalities. The marker chromosomes were characteristic of a given line and were sometimes found in multiple copies in all abnormal cells.

In the case of the cell lines, all metaphase cells were abnormal and showed the same markers as the effusion cells of the patients of origin (Table 2). Possible identification of the marker chromosomes is given in Table 3. Occasionally, minor progressive changes of a marker were observed, such as for example the marker 3q- in Me-14 effusion cells was replaced by i(3p) in the cell line Mero-14.

In the Me-25 biopsy material cultured for a few days, and in a small percentage of the mitoses from the Me-25 effusion, the karyotype was found to contain 67 chromosomes (range 65-69). However, the cell line Mero-25 contained 135 chromosomes, which was also found in the majority of the effusion cells at diagnosis.

Growth kinetics

The growth of normal mesothelial cells is promoted by the addition of EGF plus HC as described by Connell et al. (22). We could confirm these results in ^{3}H-thymidine incorporation experiments with normal mesothelial cells isolated from the pleural effusion of a non-cancerous patient (unpublished results). The growth characteristics of the malignant mesothelioma cell lines Mero-14 and Mero-25 in media with different supplements were analyzed. The addition of either EGF plus HC or HC only inhibited the growth of the malignant mesothelioma cell lines Mero-14 and Mero-25 (fig. 1). Addition of EGF only increased the proliferation slightly as compared to the growth in medium without EGF supplementation (fig. 1). The cell line Mero-41 was not tested in this assay. After its establishment, this cell line did no longer require EGF and HC for its growth.

TABLE 3

Possible cytogenetic interpretation of the markers

	Mero-14		Mero-25		Mero-41
1p-	1qter→p11::?	1p-	1qter→p11::sat	inv del (1)	?1q22→cen::1p31→p35::1q24→qter
1q-	1p::19q	inv(1q-)	inv(1)(p13p36)del(1)(q12)		
		2p+	2qter→p12::?		
3p-	del(3)(p23)			3p-	del(3)(p12)
				3q-	del(3)(q13)
3q-	3pter→q11::?	3p-	del(3)(p21)	Mar(3)	3qter→p11::?::cen::?14q-
	or sometimes i(3p)				
3p+	3qter→p21::1q11→qter				
		5q-	del(5)(q21q34)	inv(5)	inv(5)(p15q11)
6p-	6qter→cen::?				
		inv(X)	inv(X)(p22q12)		
mar(7)	7pter→cen::? 7q32→q11::19p or q				
				8p-	8qter→p11::?17p
					or sometimes i(8q)
9p+	dup(9)(p21→p22) or HSR	9p-	del(9)(p21)	9p-	del(9)(p21)
		9p+	9q::19p	9p+	dup inv(9)(p13p22)
		MarC	?inv(11)(p11q14)		
12q+	12pter→q23::3q12→qter			12p-	del(12)(p11p12)
				14q-	del(14)(q23q31)
				15q-	der(15)t(15;22)(q21;q11)
Mar(16)	?	Mar(16)	?		
				17p+	8p::17q
		inv(18)	inv(18)(p11q12)		
		MarE	?17q- or ?19q+		
		20p+	20qter→p11::21q11→qter	20q+	dup20q or der(20)t(15;20)(q?;q?)
				i(21q)	isochromosome 21q
22q+	22pter→q13::?	22q+	? duplication	22q+	der(22)t(15;22)(q21;q11)

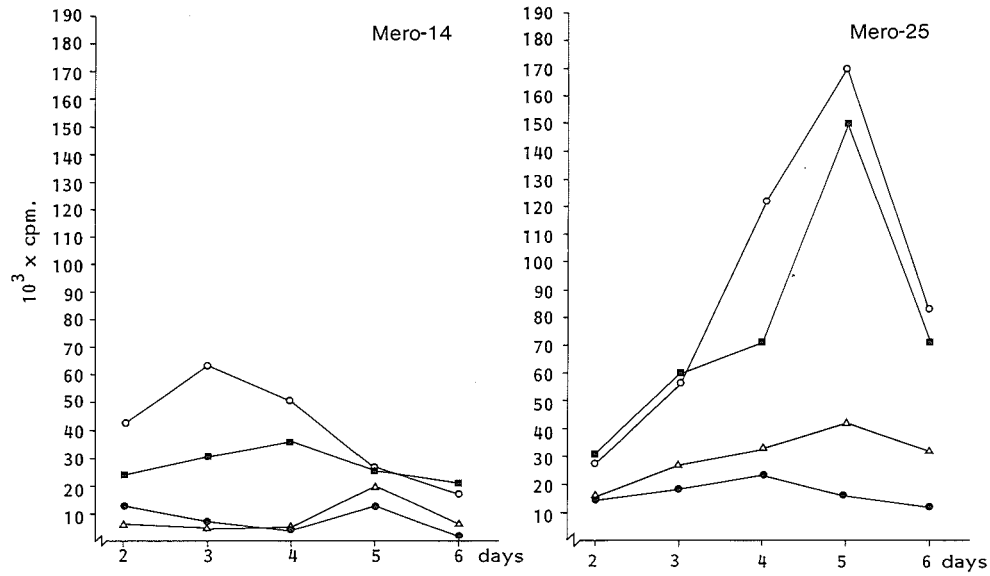

Figure 1. *^{3}H-thymidine incorporation in Mero-14 (left) and Mero-25 (right). Cell lines were cultured in F10 medium with 15% FCS and different supplements. ■ F10 medium with 15% FCS; ○ F10 medium, 15% FCS, and 10 ng/ml EGF; △ F10 medium, 15% FCS, 10 ng/ml EGF, and 0.4 µg/ml HC; ● F10 medium, 15% FCS and 0.4 µg/ml HC.*

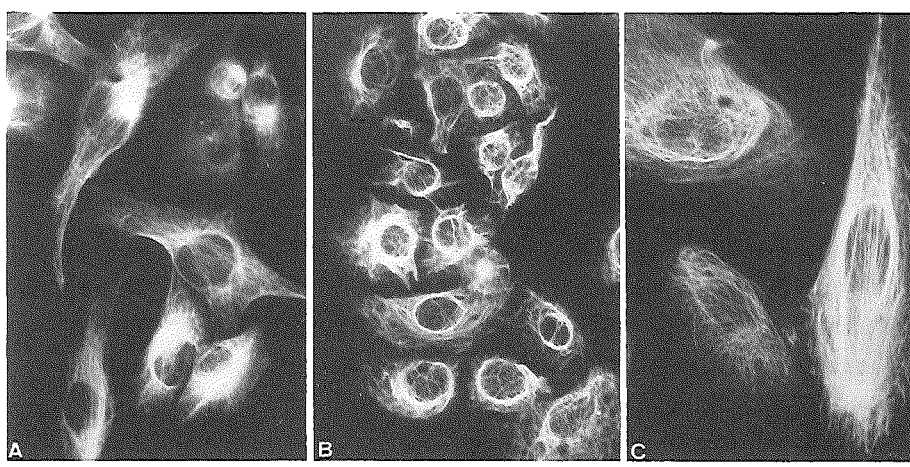

Figure 2. *Detection of cytokeratins in Mero-14(a), Mero-25(b), and Mero-41(c) by indirect immunofluorescence using the polyclonal cytokeratin antibody pKer. a-c) x 500.*

Intermediate filament protein expression

In order to support the mesothelial nature of the isolated cell lines and to investigate whether Mero-14, Mere-25 and Mero-41 contained intermediate filament proteins (IFP) typical of mesothelioma (7), several antibodies to IFP were applied in the indirect immunofluorescence assay and in immunoblotting tests. Table 1 and Figures 2-4 summarize the results of these studies.

When the polyclonal rabbit antiserum to skin keratins was applied to methanol fixed cells, Mero-14, Mero-25 and Mero-41 all showed filamentous staining reactions, albeit in different patterns and intensities (compare Figs. 2 a, b, and c). The same holds true for the broadly cross-reacting monoclonal antibody RCK102.

When the monoclonal antibodies, each specific for only one cytokeratin polypeptide, were applied to the cell lines, it became obvious that all three cell lines contained cytokeratin 18 and cytokeratin 7 in virtually all cells.

When monoclonal antibody LP2K, specifically reacting with cytokeratin 19, was applied to Mero-14, only about 5% of the cells were found to show a filamentous staining reaction. Mero-25 and Mero-41 showed a filamentous reaction in virtually all cells present in the culture. Furthermore, all cells showed filamentous staining reactions when incubated with the polyclonal or monoclonal antibody to bovine lens vimentin (pVIM and RV202, respectively), but again typical differences in the distribution patterns and staining intensity were found. No reactions were seen when antibodies to cytokeratin 10 (RKSE 60), desmin (RD301), or only second step antibodies were applied (see Table 1).

Cytoskeletal preparations of the different cell lines were analyzed by one- and two-dimensional gel electrophoresis as well as by immunoblotting assays. The one-dimensional gels of these cytoskeletal preparations revealed protein bands at the molecular weight levels of vimentin, actin, and cytokeratins 7, 8, 18 and 19 (Fig. 3, lanes 1), however, in varying amounts. For example, in Mero-14, the vimentin protein band was most pronounced, while in Mero-25 and Mero-41, this IFP occurred in concentrations comparable to those of the cytokeratins. Two-dimensional gel electrophoresis (Figs. 4 a, g, and j) confirmed these findings.

The individual protein bands were identified using the Western blotting technique in combination with the specific monoclonal antibodies. In all three cell lines cytokeratins 18, 8, 7 and 19 as well as vimentin were detected with the antibodies RCK106, RCK102, RCK105, LP2K and RV202, respectively (see Fig. 3). RCK102 also reacted with a protein band migrating just below vimentin in the one-dimensional immunoblots of all three cell lines (Fig. 3, lanes 3). This antibody has been shown previously in immunoblotting studies on keratin preparations of human epidermis and from human squamous cell carcinomas to recognize cytokeratin 5 next to cytokeratin

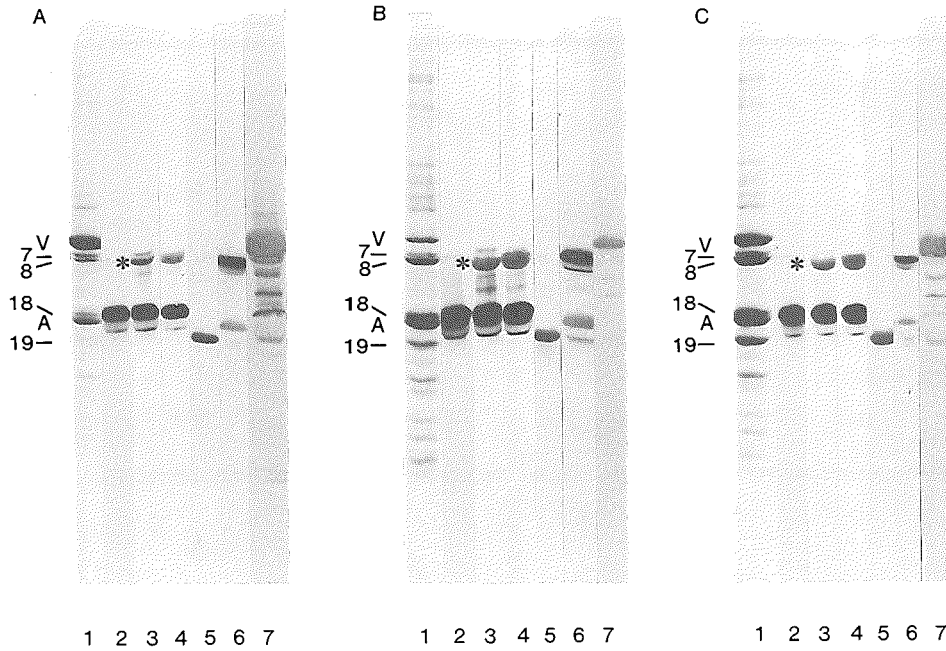

Figure 3. *One-dimensional SDS-containing polyacrylamide gel electrophoresis and subsequent protein immunoblotting tests of cytoskeletal preparations from Mero-14 (panel A), Mero-25 (panel B), and Mero-41 (panel C). lanes 1: Coomassie brilliant blue stained gels; lanes 2: protein blots incubated with RCK106 (anti-cytokeratin 18); lanes 3: lanes 2, subsequently incubated with RCK102 (anti-cytokeratin 5 + 8); the asterisk indicates immunoreactivity with the extra protein band between vimentin and cytokeratin 8; lanes 4: lanes 3, subsequently incubated with RCK 105 (anti-cytokeratin 7). lanes 5: protein blots incubated with LP2K (anti-cytokeratin 19); lanes 6: protein blots incubated with RCK105 (anti-cytokeratin 7). The immunoreaction at the level of actin is probably caused by the presence of breakdown products of cytokeratin 7; lanes 7: protein blots incubated with RV202 (anti-vimentin). Note that the antibody also reacts with the vimentin breakdown products. Abbreviations: v : vimentin; a : actin; 7, 8, 18, and 19 indicate the corresponding cytokeratin polypeptides.*

8. Although the immunostained protein band migrates in the position of cytokeratin 5, two-dimensional immunoblotting could not confirm the occurrence of this cytokeratin polypeptide in the mesothelioma cell lines. Two-dimensional immunoblots, which were subsequently incubated with antibodies RCK106, RCK102, RCK105, RV202, and LP2K, confirmed the one-dimensional immunoblotting studies with respect to cytokeratins 18, 8, 7, and 19 (Fig. 4).

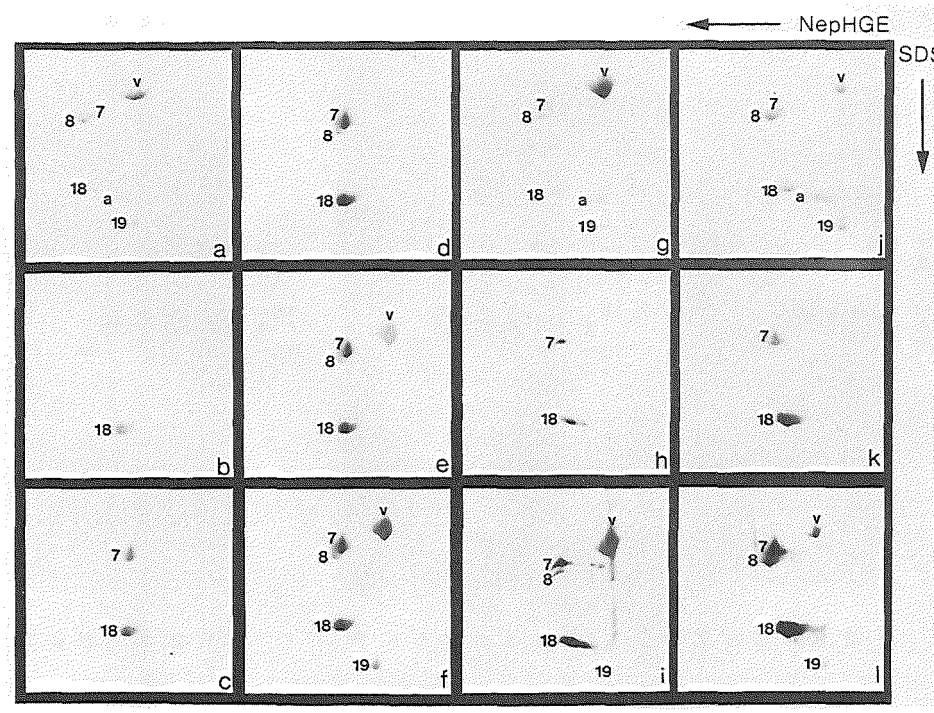

Figure 4. *Two-dimensional gel electrophoresis (a,g,j) and immunoblotting assays of these gels (b-f,h,i,k,l) of cytoskeletal preparations from cell lines Mero-41 (a-f), Mero-14 (g-i), and Mero-25 (j-l). The protein blots were subsequently incubated with RCK106 (b,h,k), RCK105 (c,h,k), RCK102 (d,i,l), RV202 (e,i,l) and LP2K (f,i,l). Abbreviations: v : vimentin; a : actin; 7,8,18 and 19 indicate the corresponding cytokeratin polypeptides; NEpHGE, first-dimension non-equilibrium pH gel electrophoresis; SDS, second dimension SDS polyacrylamide gel electrophoresis.*

DISCUSSION

Three continuously growing cell lines have been derived *in vitro* from untreated malignant mesothelioma cells from three different patients. The malignant mesothelioma character of the three cases has been demonstrated cytologically, histologically and/or ultrastructurally.

The isolated cell lines show individual cellular morphology and growth characteristics. Cytomorphologic and ultrastructural studies showed that these cell lines

have characteristics consistent with mesothelioma and definitely different from that of adenocarcinomas.

Cytogenetic studies demonstrated that the cell lines shared the same chromosomal aberrations as the primary tumor cells obtained from the patient material and that they thus represented *in vitro* proliferation of the same tumor. Although no chromosomal aberrations specific for malignant mesothelioma could been identified on the basis of this study, the nature of the abnormalities observed in the cell lines follow the same pattern of monosomy and marker formation as observed in a large series of mesothelioma patients that we have studied: two types of clonal changes were (a) most frequently a hypodiploid/hypotetraploid karyotype characterized by relative loss of chromosomes 4, 6, 13, and 22 or (b) an hyperdiploid karyotype with among other changes extra copies of chromosome 7 (A. Hagemeijer et al., manuscript in preparation). Gibas et al. (6) investigated the karyotype of 14 patients with a malignant mesothelioma and suggested the nonrandom involvement of chromosomes 1, 2, 3, 6, 9, 11, 17, and 22 in this tumor. In the three malignant mesothelioma cell lines described in this study we found structural abnormalities of chromosomes 1, 3, 9, and 22 (Table 3). Other markers were only occasionally present in these cell lines and in other mesotheliomas studied. This may be due to the fact that patients Me-14, Me-41, and Me-25 had not received therapy at the time that the pleural effusions were collected. Two recent publications on cytogenetics of malignant mesothelioma also emphasize the absence of specific changes (23,24). Structural abnormalities of chromosome 3 reported by Popescu et al. (23) are consistent with our findings.

Lechner et al. (25) reported that asbestos treatment of normal mesothelial cell cultures induced hypodiploidy and selective loss of chromosomes 11 and 21. In cell lines Mero-14, Mero-25, and Mero-41, however, the chromosomes 11 and 21 were present in numbers that might be expected in these cells.

Connell and Rheinwald (22) described the stimulating effect of EGF plus HC supplements on the proliferative capacity of normal mesothelial cells, whereas addition of EGF or HC only did not promote the growth of the normal mesothelial cells. In contrast to the observation in normal mesothelial cells the growth of the cell lines Mero-14 and Mero-25 was found to be inhibited by the addition of EGF plus HC in similar concentrations. This negative effect appeared to be caused by HC, as the addition of HC only decreased the proliferation of Mero-14 and Mero-25, whereas the addition of EGF slightly increased their growth rate.

Extensive studies on intermediate filament protein expression support the assumption that Mero-14, Mero-25 and Mero-41 are derived from mesotheliomas and have retained this character. First of all, the detection of cytokeratins in all three cell lines proves their epithelial nature. The fact that the cell lines additionally express

vimentin also seems to be in support of the mesothelial derivation, because normal and malignant mesothelial cells have been shown to be able to coexpress cytokeratins and vimentin *in vivo* (8). However, one should realize that many cell types initiate the synthesis of vimentin when brought into tissue culture (26).

Further proof of the mesotheliomal nature of the three cell lines has come from the study of their individual cytokeratin polypeptides. Blobel et al. (7), showed that mesotheliomas can contain cytokeratins 7, 8, 18, and 19 and occasionally some other cytokeratins. Especially the presence of cytokeratin 5 in some of these tumors is noteworthy in this respects because normal mesothelial cells apparently do not express this cytokeratin polypeptide and contain only cytokeratins 7, 8, 18, and 19 (27-30). Aside from our findings that the cell lines contain cytokeratins 7, 8, 18 and 19, based on immunofluorescence and immunoblotting tests with chain specific monoclonal cytokeratin antibodies, we were not able to confirm the presence of a cytokeratin 5-like protein using two dimensional gel electrophoresis and immunoblotting.

Connell and Rheinwald (22) found that during rapid growth, normal mesothelial cells reduce cytokeratin levels, elevate vimentin expression, and adopt a fibroblastoid shape. Reduction of the growth rate, on the other hand, would result in increase of the keratin content, decrease of vimentin, and an epitheloid phenotype of the cells. In the malignant mesothelioma cell lines Mero-14, Mero-25, and Mero-41, keratin and vimentin expression appeared to be present during rapid growth as well as in confluent cultures. The cell line with the highest vimentin:keratin ratio (Mero-14) had the highest proliferation rate, fibroblastoid shaped cells, and in transmission electron microscopy, no villi could be distinguished, in contrast to Mero-25 and Mero-41. The observed correlation between the vimentin:cytokeratin ratio, proliferation rate, and phenotype in normal mesothelial cells seems to hold true for malignant mesothelioma cell lines as well. However, in contrast to normal mesothelial cells, the vimentin:cytokeratin level and the phenotype of the investigated malignant mesothelioma cell lines appeared to be typical for each cell line and could not be altered by affecting the growth rate.

In summary, characterization of the cell lines support the assumption that Mero-14, Mero-25 and Mero-41 are derived from malignant mesotheliomas and have retained this character. They exhibit features of mesothelial cells such as ultrastructural organization and intermediate filament protein expression. Their response to growth factors is different from that of benign mesothelial cells, as is the highly abnormal karyotype, and the latter has been associated with malignant mesothelioma (6).

Establishment of tumor cell lines has notably extended the possibilities for cell biologic and genetic investigations of tumors. Malignant mesothelioma is a rare tumor for which viable tissue specimens are difficult to obtain, and the availability of

characteristic cell lines is therefore particularly important. To our knowledge only a few malignant mesothelioma cell lines have been described (31-33). The use of such cell lines should promote research aiming at the study of the mechanism of oncogenesis of this particular cancer, and development of specific diagnostic tools, which are needed for better diagnosis and safer therapeutic decisions in the clinical management of patients presenting with pleural effusions.

ACKNOWLEDGMENTS

This study was in part supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds). The authors gratefully acknowledge Prof.Dr. R. Benner for his continuous support. Dr. J.J.M. van Dongen, Dr. H. Hooijkaas, Ms. E.M.E. Smit, Ms. E. Schoenmaker, Ms. M. Moret, Ms. I.L.M. Wolvers-Tettero, Mr. O. Moesker and Ms. A. Huijsmans for their advice and/or excellent technical assistance. The lung physicians in the area of Rotterdam and Vlissingen are acknowledged for providing patient material for this study. Dr. E.B. Lane (I.C.R.F., London) is acknowledged for kindly providing the monoclonal antibody LP2K and Ms. J. de Goey-van Dooren for typing the manuscript.

REFERENCES

1. Wagner JC, Sleggs CA, Marchand P (1960): Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. Br J Ind Med 17:260-271.
2. Whitaker D, Shilkin KB (1984): Diagnosis of pleural malignant mesothelioma in life - a practical approach. J Pathol 143:147-175.
3. Van der Kwast TH, Versnel MA, Delahaye M, Jong de A, Zondervan PE, Hoogsteden HC (1988): Expression of epithelial membrane antigen on malignant mesothelioma cells: an immunocytochemical and immunoelectron microscopic study. Acta Cytol: 169-174.
4. Kobzik L, Antman KH, Warhol MJ (1985): The distinction of mesothelioma from adenocarcinoma in malignant effusions by electron microscopy. Acta Cytol 29:219-225.
5. Kwee W-S, Veldhuizen RW, Alons CA, Morawetz F, Boon ME (1982): Quantitative and qualitative differences between benign and malignant mesothelial cells in pleural fluid. Acta Cytol 26:401-406.
6. Gibas Z, Li PF, Antman KH, Bernal S, Stahel R, Sandberg AA (1986): Chromosome changes in malignant mesothelioma. Cancer Genet Cytogenet 20:191-201.
7. Blobel GA, Moll R, Franke WW, Kayser KW, Gould VE (1985): The intermediate filament cytoskeleton of malignant mesotheliomas and its diagnostic significance. Am J Pathol 121:235-247.
8. LaRocca PJ, Rheinwald JG (1984): Coexpression of simple epithelial keratins and vimentin by human mesothelium and mesothelioma *in vivo* and in culture. Cancer Res 44:2991-2999.
9. Said JW, Nash G, Banks-Schlegel S, Sassoon AF, Shintaku IP (1984): Localized fibrous mesothelioma : An immunohistochemical and electron microscopic study. Hum Pathol 15:440-443.
10. Czernobilsky B, Moll R, Levy R, Franke WW (1985): Co-expression of cytokeratin and vimentin filaments in mesothelial, granulosa and rete ovarii cells of the human ovary. Eur J Cell Biol 37:175-190.
11. Holthöfer H, Miettinen A, Paasivuo R, Lehto V-P, Linder E, Alfthan O, Virtanen I (1983): Cellular origin and differentiation of renal carcinomas : A fluorescence microscopic study with kidney-specific antibodies, antiintermediate filament antibodies, and lectins. Lab Invest 49:317-326.
12. Ramaekers FCS, Haag D, Kant A, Moesker O, Jap PHK, Vooijs GP (1983): Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. Proc Natl Acad Sci USA 80:2618-2622.
13. Ramaekers F, Haag D, Jap P, Vooijs PG (1984): Immunochemical demonstration of keratin and vimentin in cytologic aspirates. Acta Cytol 28:385-392.
14. Gatter KC, Dunnill MS, van Muijen GNP, Mason DY (1986): Human lung tumours may coexpress different classes of intermediate filaments. J Clin Pathol 39:950-954.
15. Versnel MA, Bouts MJ, Hoogsteden HC, Van der Kwast ThH, Delahaye M, Hagemeijer A (1989): Establishment of human malignant mesothelioma cell lines. Int J Cancer: in press.
16. Ramaekers FCS, Puts JJG, Moesker O, Kant A, Huysmans A, Haag D, Jap PHK, Herman CJ, Vooijs GP (1983): Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours: An overview. Histochem J 15:691-713.

17. Ramaekers F, Huysmans A, Moesker O, Kant A, Jap P, Herman C, Vooijs P (1983): Monoclonal antibody to keratin filaments, specific for glandular epithelia and their tumors. Use in surgical pathology. Lab Invest 49:353-361.
18. Ramaekers F, Huysmans A, Schaart G, Moesker O, Vooijs P (1987): Tissue distribution of keratin 7 as monitored by a monoclonal antibody. Exp Cell Res 170:235-249.
19. Lane EB, Bártek J, Purkis PE, Leigh IM (1985): Keratin antigens in differentiating skin. Ann NY Acad Sci 455:241-258.
20. Broers JLV, Carney DN, Klein Rot M, Schaart G, Lane EB, Vooijs GP, Ramaekers FCS (1986): Intermediate filament proteins in classic and variant types of small cell lung carcinoma cell lines: A biochemical and immunochemical analysis using a panel of monoclonal and polyclonal antibodies. J Cell Sci 83:37-60.
21. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R (1982): The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 31:11-24.
22. Connell ND, Rheinwald JG (1983): Regulation of the cytoskeleton in mesothelial cells: Reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell 34:245-253.
23. Popescu NC, Chahinian AP, DiPaolo JA (1988): Nonrandom chromosome alterations in human malignant mesothelioma. Cancer Res 48:152-147.
24. Tiainen M, Tammilehto K, Mattson K, Knuutila S (1988): Nonrandom chromosomal abnormalities in malignant pleural mesothelioma. Cancer Genet Cytogenet 33:251-274.
25. Lechner JF, Tokiwa T, LaVeck M, Benedict WF, Banks-Schlegel S, Yeager H, Banerjee A, Harris CC (1979): Asbestos-associated chromosomal changes in human mesothelial cells. Proc Natl Acad Sci USA 82:3884-3888.
26. Franke WW, Schmid E, Winter S, Osborn M, Weber K (1985): Widespread occurrence of intermediate-sized filaments of the vimentin-type in cultured cells from diverse vertebrates. Exp Cell Res 123:25-46.
27. Quinlan RA, Schiller DL, Hatzfeld M, Achtstäter T, Moll R, Jorcano JL, Magin TM, Franke WW (1985): Patterns of expression and organization of cytokeratin intermediate filaments. Ann NY Acad Sci 455:282-306.
28. Moll R, Levy R, Czernobilsky B, Hohlweg-Majert P, Dallenbach-Hellweg G, Franke WW (1983): Cytokeratins of normal epithelia and some neoplasms of the female genital tract. Lab Invest 49:599-610.
29. Wu Y-J, Parker LM, Binder NE, Beckett MA, Sinard JH, Griffiths CT, Rheinwald JG (1982): The mesothelial keratins: A new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. Cell 31:693-703.
30. Rheinwald JG, O'Connell TM, Connell ND, Rybak SM, Allen-Hoffman BL, La Rocca PJ, Wu Y-I, Rehwoldt SM (1984): Expression of specific keratin subsets and vimentin in normal human epithelial cells : A function of cell type and conditions of a growth during several culture. In: Cancer Cells, 1, The Transformed Phenotype. AJ Levine, GF Vande Woude, WC Topp, JD Watson eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 217-277.
31. Behbani AM, Hunter WJ, Chapman AL, Lin F (1982): Studies of a human mesothelioma. Hum Pathol 13:862-866.
32. Shanfang W, Enzhong W, Jianzhong S, Huixuan X, Zhenqiong L, Cifu S, Changwen X, Ruiming C, Xiuzhen Y, Dehou Z, Zhenhong Y (1985): Establishment and characterization of human malignant pleural mesothelioma cell line SMC-1. Sci Sin 28:281-288.
33. Hsu S-M, Hsu P-L, Zhao X, Kao-Shan CS, Whang-Peng J (1988): Establishment of human mesothelioma cell lines (MS-1, -2) and production of a monoclonal antibody (Anti-MS) with diagnostic and therapeutic potential. Cancer Res 48:5228-5236.

CHAPTER 7

EXPRESSION OF PDGF AND PDGF RECEPTORS IN HUMAN MALIGNANT MESOTHELIOMA

7.1 Epression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors

7.2 Expression of the c-*sis* oncogene in pleural effusion cells and tumor material of human malignant mesotheliomas

7.3 Expression of PDGF receptors in human malignant mesothelioma cell lines and cultured normal mesothelial cells

CHAPTER 7.1

EXPRESSION OF C-SIS (PDGF B-CHAIN) AND PDGF A-CHAIN GENES IN TEN HUMAN MALIGNANT MESOTHELIOMA CELL LINES DERIVED FROM PRIMARY AND METASTATIC TUMORS

Marjan A. Versnel[1], Anne Hagemeijer[1], Monique J. Bouts[1], Theo. H. van der Kwast[2] and Henk C. Hoogsteden[3]

Department of Cell Biology, Immunology and Genetics[1], Department of Pathology[2] and Department of Pulmonary Medicine[3], Erasmus University and Academic Hospital Dijkzigt, Rotterdam, The Netherlands

Oncogene 2, 601-605, 1988.

ABSTRACT

Ten human malignant mesothelioma cell lines from primary and metastatic sites were studied for the expression of c-*sis* (PDGF B-chain) and PDGF A- chain genes. Malignant mesothelioma cell lines expressed strongly the c-*sis* oncogene which is barely detectable in normal mesothelial cells. The PDGF A-chain gene expression was slightly elevated in malignant mesothelioma cell lines compared to the expression in normal mesothelial cells. Cytogenetic and Southern blot analysis did not provide evidence for genomic amplification or rearrangement of the c-*sis* oncogene. These results suggest that malignant mesothelioma cell lines show constitutively enhanced expression of the c-*sis* and PDGF A-chain genes that could play a role in the etiology of this type of malignancy.

INTRODUCTION

Exogenous growth factors control the proliferation of normal diploid cells in culture. Malignant cells in culture are often independent of exogenous growth factors (Holley, 1975). This independence can be explained by the autocrine production of growth factors or truncated growth factor receptors (Todaro and De Larco, 1978; Sporn and Todaro, 1980; Heldin and Westermark, 1984). A direct relationship between growth factors and oncogenes has been observed for the platelet-derived growth factor (PDGF) and the oncogene v-*sis* of Simian Sarcoma Virus (SSV). The amino acid sequence of the B-chain of human PDGF has been found to be nearly identical to the oncogene product $p28^{sis}$ (Waterfield et al., 1983; Doolittle et al., 1983). Moreover in SSV transformed cells a PDGF-like growth factor has been detected (Deuel et al., 1983). PDGF is mitogenic to cells with functional PDGF receptors like connective tissue cells. The human c-*sis* (PDGF B) oncogene has been mapped to chromosome 22 (Swan et al., 1982; Dalla Favera, 1982). The PDGF A-chain was recently cloned and mapped to chromosome 7p22-p21 (Betsholtz et al.; 1986, Morton et al., 1987).

The single mRNA transcript from the c-*sis* gene has been found in several normal cell types like endothelial cells, cytotrophoblasts and activated macrophages (Barrett et al., 1984; Goustin et al., 1985; Martinet et al., 1986). Expression of the c-*sis* mRNA has also been found in several human tumor cell lines like osteosarcomas, glioblastomas, fibrosarcomas and occasionally in melanomas (Eva et al., 1982; Pantazis et al., 1985; Graves et al., 1984; Westermark et al., 1986). In some of these cell lines secretion of PDGF-like proteins has been detected (Betsholtz et al., 1983; Betsholtz et al., 1984; Nister et al., 1984). A general problem in these studies is that

little is known about the expression of c-*sis* in the normal counterparts of these tumor cells.

The diagnosis of malignant mesothelioma is difficult since there are no morphologic criteria for differentiation between benign and malignant mesothelial cells and adenocarcinoma cells (Whitaker et al., 1984). The incidence of malignant mesotheliomas has a strong relationship to asbestos exposure (Wagner et al., 1960). Gerwin et al. (1987a, b) have recently detected the expression of c-*sis* in several malignant mesothelioma cell lines. We investigated the expression of the c-*sis* and PDGF A-chain genes in a panel of ten malignant mesothelioma cell lines derived from primary and metastatic tumor sites. Control mesothelial cells were also studied. All cell lines were karyotyped and Southern blot analysis of c-*sis* was performed in six cell lines.

MATERIALS AND METHODS

Cell lines and growth conditions

Ten human malignant mesothelioma cell lines, Mero-14, -25, -41, -48a, - 48b, -48c, -48d, -72, -82 and -84, were isolated from pleural effusions, biopsy or autopsy material as described previously (Versnel et al., submitted). All malignant mesothelioma cell lines were isolated from patients with cytologically, histologically and/or ultrastructurally confirmed malignant mesotheliomas. Cytological and ultrastructural morphology of the malignant mesothelioma cell lines was consistent with their mesothelial origin and expression of vimentin and cytokeratins was found. Several of the cell lines have been maintained *in vitro* for more than 50 passages without showing signs of senescence. The cell line Mero-48a was derived from a primary tumor (pleura), while -48b, -48c and -48d cell lines were isolated from metastatic sites (omentum, liver and pericard) at autopsy of a mesothelioma patient. The malignant mesothelioma cell lines were cultured in Ham's F10 medium (Gibco, Paisley, United Kingdom) supplemented with 15% fetal calf serum (FCS).

Five normal mesothelial cell cultures were established from pleural effusions or biopsy material from patients without a malignant mesothelioma. Cytologically these cells were recognized as mesothelial cells and expression of vimentin and cytokeratins was found. The karyotype of the normal mesothelial cell cultures was normal diploid and they had a limited lifespan of approximately fifteen passages. The cells were cultured in the same medium as described above supplemented in addition with 10 ng/ml Epidermal Growth Factor (EGF, Collaborative Research Inc., Lexington, M.A.) and 0.5 μg/ml Hydrocortisone (HC).

Both cell types were routinely subcultured two or three times a week.

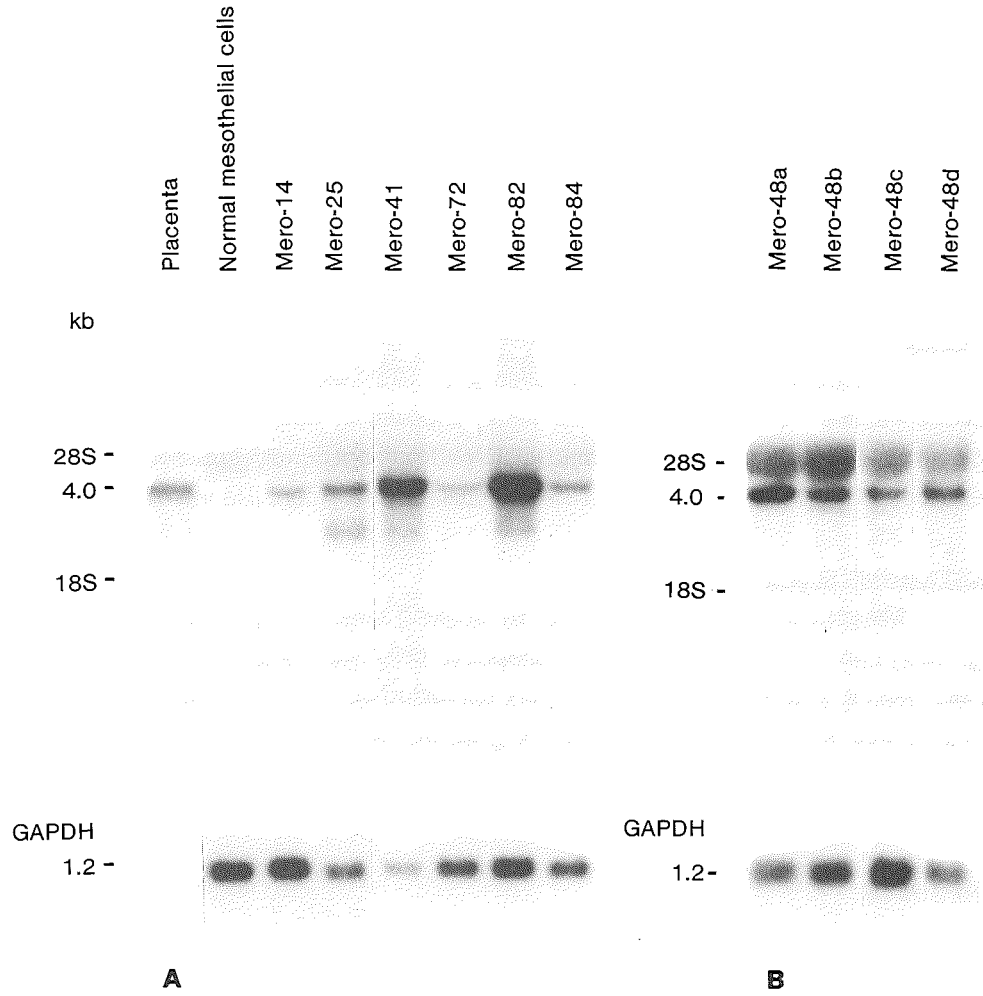

Figure 1. *Northern blot analysis of 25 µg total RNA of placenta, normal mesothelial cells and the malignant mesothelioma cell lines hybridized to ^{32}P-labeled c-sis and GAPDH probes.*

RNA isolation and Northern blot analysis

Cells were harvested with a rubber policeman and homogenized in an Omni-mixer (Du Pont, Newtown, C.T.). Total RNA was isolated using LiCl/Urea method (Auffray and Rougeon, 1980). Electrophoresis of 20 or 25 µg total RNA was performed on a 1% agarose gel with formaldehyde (Maniatis et al., 1982). As marker an RNA ladder was used (BRL; Gaithersburg, MD). After blotting to nitrocellulose (Thomas et al., 1980) hybridization was performed according to Jeffreys and Flavell (1977). Filters were washed at 65°C to 0.1 x SSC and exposed to a Fuji-RX film.

DNA isolation and Southern blot analysis

DNA isolation was performed according to the method described by Jeffreys and Flavell (1977). Restriction enzyme-digested DNAs were electrophoresed on 0.7% agarose gels and were transferred to Gene Screen Plus filters (New England Nuclear, Boston, M.A.). Hybridization to ^{32}P-labeled probes was performed according to Bernards and Flavell (1980).

Probes

A 1.7 *Bam*H1 c-*sis* fragment (Groffen et al., 1983) was used. The PDGF A- chain probe was the 1.3 kb *Eco*R1 fragment kindly supplied by Betsholtz et al. (1986). The *bcr* probe was a 3' 1.2 kb *Hin*dIII-*Bgl* DNA fragment kindly supplied by Dr. G. Grosveld. The GAPDH probe was a 0.7 kb *Eco*RI-*Pst*1 fragment (Benham et al., 1984).

Cytogenetic analysis

Metaphase cells were obtained by shaking culture flasks with exponentially growing cells. The supernatant with metaphase cells was collected and after 15 min colcemid incubation the metaphases were harvested according standard cytogenetic procedures. RFA-, QFQ- and GTG-banding techniques were used and the karyotype was established according to the I.S.C.N. (1985).

RESULTS

Expression of c-*sis* and PDGF A-chain mRNA in mesothelioma cell lines

RNA was isolated from ten human malignant mesothelioma cell lines and five cultured normal mesothelial cell lines. Placental RNA was used as a positive control for c-*sis* transcripts and showed a band of 4.0 kb. All human malignant mesothelioma cell lines showed a 4.0 kb mRNA that strongly hybridized to the c-*sis* probe (figure 1, A and B). In Mero-25 and Mero-41 a weak cross hybridization of the c-*sis* probe with the 2.3 kb PDGF A-chain transcript was detected. In normal mesothelial cells no signal was detected (figure 1A). It is only after long exposure (5 - 10 days) of 10 μg poly(A)$^+$ RNA that faint c-*sis* specific transcripts were detected in normal mesothelial cells (data not shown). The level of c-*sis* transcription varied between the malignant mesothelioma cell lines but was always elevated. In the malignant mesothelioma cell lines isolated from primary and metastatic sites from the same patient (Mero-48a, -48b, -48c and -48d) c-*sis* transcripts were present in approximately equal amounts (figure 1B). Northern blot analysis with a PDGF A-chain probe showed expression of the 2.8, 2.3 and 1.9 kb

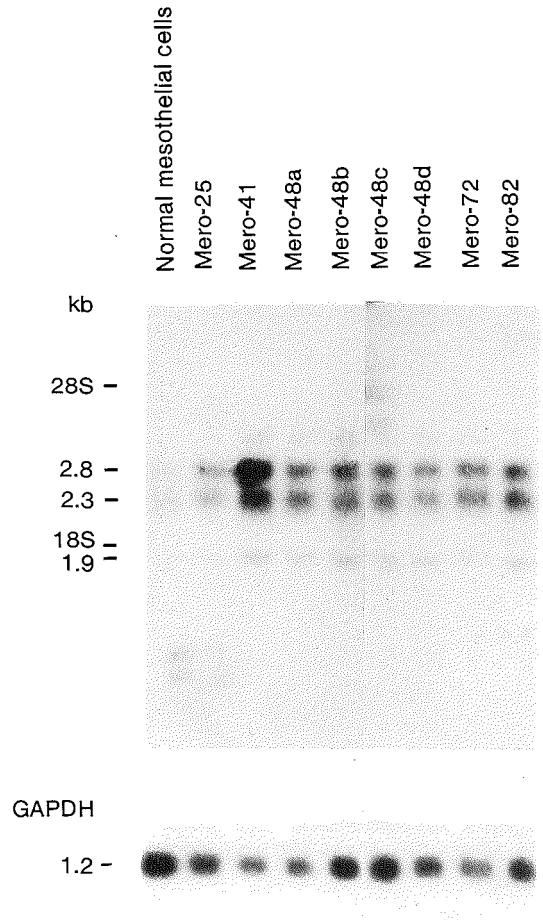

Figure 2. *Northern blot analysis of 20 µg total RNA of normal mesothelial cells and the malignant mesothelioma cell lines hybridized to ^{32}P-labeled PDGF A-chain and GAPDH probes.*

PDGF A-specific transcripts in malignant mesothelioma cell lines, normal mesothelial cells and placenta. The level of expression of the PDGF A-chain gene was elevated in malignant mesothelioma cell lines compared to normal mesothelial cells. PDGF A-chain transcripts from the cell lines Mero-25, Mero-41, Mero-48a, Mero-48b, Mero-48c, Mero-48d, Mero-72, Mero-82 and normal mesothelial cells are shown in figure 2. The quality and the amounts of RNA applied were controlled by rehybridization of the filters to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. All lanes from

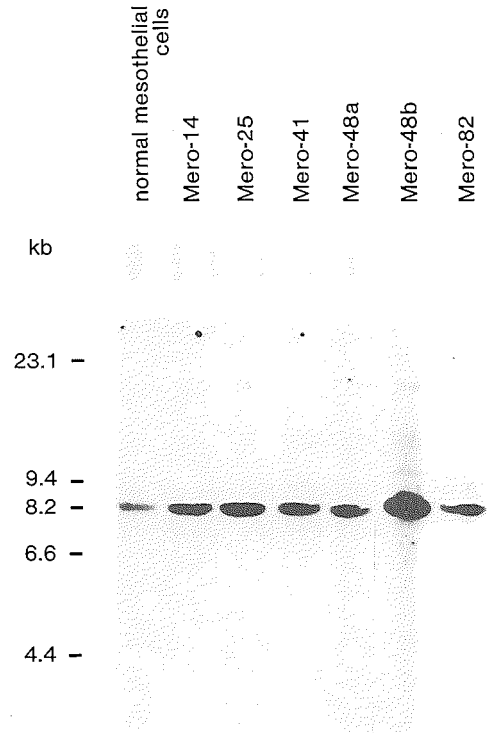

Figure 3. *Southern blot of Xba1 digested DNA's of normal mesothelial cells and the malignant mesothelioma cell lines Mero-14, Mero-25, Mero-41, Mero-48a, Mero-48b, Mero-48c and Mero-48d hybridized to a ^{32}P-labeled c-sis probe.*

cultured cell lines showed approximately equal amounts of RNA (figures 1A, 1B and 2). In placental RNA it is only after a long exposure (2 days) that a GAPDH transcript was detected. This observation is consistent with the reported difference in GAPDH expression found between cultured cells and tissues (Benham et al., 1984).

Cytogenetic aberrations in malignant mesothelioma cell lines

The c-*sis* and PDGF A-chain gene have been mapped to chromosomes 22q13.1 and 7p21-22, respectively. All ten human malignant mesothelioma cell lines showed highly abnormal karyotypes with modal chromosome numbers ranging from 38 to 75 and were characterized by the presence of numerous markers. Findings concerning ploidy and chromosomes #7 and #22 are given in table 1. No constant structural rearrangements of chromosomes 7 and 22 were observed.

TABLE 1

Cytogenetic data concerning chromosomes #7 and #22 of 10 malignant mesothelioma cell lines

Cell line	modal number of chromosomes	number of copies of chromosome			
		normal #7	rearranged #7	normal #22	rearranged #22
Mero-14	75	2	[7p-->cen::inv7 q32q11::19p]	1	1[22pter-->q13::?]
Mero-25	67	2	none	1-2	1[22q+]
Mero-41	74	4	none	3	2[der(22)t(15;22)(q21;q11)]
Mero-48a	71-75	3	1[7q-]	2	none
Mero-48b	71-75	2	2[7q-]	3	none
Mero-48c	71-75	2	1[7q-]	3	none
Mero-48d	71-75	2	1[7q-]	2	none
Mero-72	42	2	none	1	1[22q-]
Mero-82	49	2	1[7p::22q]*	2	1[7p::22q]*
Mero-84	38	2	none	1	none

* Same marker chromosome.

The number of copies of 22 (normal and involved in marker chromosomes) were usually equal to or lower than the average number of other autosomes, while the number of copies of 7p was equal or slightly higher than the average number of other autosomes: e.g. 4 copies of 7 in hypotetraploid karyotypes of Mero-41 and Mero-48a and -48b. The karyotype of the cultured mesothelial cells was normal diploid.

Southern blot analysis of the c-*sis* gene in malignant mesothelioma cell lines

DNA from normal mesothelial cells and six malignant mesothelioma cell lines (Mero-14, Mero-25, Mero-41, Mero-48a, Mero-48b and Mero-82) was investigated for rearrangements in the c-*sis* gene. In *Xba* I digested DNA's the c-*sis* probe hybridized to a band of 8.2 kb (figure 3). Digestion of malignant and normal cell DNA with two other restriction enzymes (*Eco*R1 and *Hin*dIII) revealed also the specific germline bands and no rearranged fragments (data not shown). The filters with *Xba*I, *EcoR*1 and *Hin*dIII digested DNA were rehybridized with *bcr* and c-*sis* probes (data not shown). The results indicate absence of genomic amplification or loss of copies of c-*sis*. The difference in band intensities in figure 3 and the chromosomal counts in table 2 are caused by difference in loading and was not consistently present in the other digests and correlated with the *bcr* bands.

DISCUSSION

Expression of the c-*sis* (PDGF B-chain) and PDGF A-chain was demonstrated in ten human malignant mesothelioma cell lines from primary and metastatic tumor. Malignant mesothelioma cell lines expressed strongly the c-*sis* oncogene which is barely detectable in normal mesothelial cells. Gerwin et al. (1987 a,b) first reported expression of PDGF A- and B-chains in human malignant mesothelioma cell lines. They found high levels of either PDGF A- or PDGF B-chain message or both in malignant mesothelioma cell lines. Within our panel of malignant mesothelioma cell lines we found variable though elevated amounts of c-*sis* transcripts, while little variation in the PDGF A-chain expression was observed.

All the human malignant mesothelioma cell lines so far investigated expressed the c-*sis* oncogene. This is in contrast with the findings in human osteosarcoma and melanoma cell lines where c-*sis* expression is not a consistent feature of all tumor cell lines investigated (Eva et al., 1982; Betsholtz et al., 1986; Westermark et al., 1986). Westermark et al. (1986) reported that the c-*sis* expression in a human melanoma cell line from a primary tumor was highly elevated compared to cell lines from metastases. In our study, malignant mesothelioma cell lines from primary and metastatic tumors showed no significant differences in the level of c-*sis* expression. Moreover, preliminary results suggest that c-*sis* expression was not restricted to malignant mesothelioma cell lines in culture, but can also be detected in total RNA isolated from pleural effusions and autopsy material of malignant mesothelioma patients.

To investigate the mechanism of c-*sis* activation the malignant mesothelioma cell lines were studied for chromosomal aberrations of chromosome 22 and DNA rearrangements in the c-*sis* gene. Although aberrations in chromosome 22 were detected in several of the malignant mesothelioma cell lines there was no specific marker present in all the investigated cell lines. The c-*sis* expression can not be due to an elevated copy number of the c-*sis* containing chromosome as several cell lines have only two copies of chromosome 22. Restriction enzyme analysis with different enzymes did not reveale a rearrangement in the c-*sis* gene and hybridization with a *bcr* probe indicated an absence of genomic amplification of the c-*sis* gene. There were no indications that rearrangements on DNA or chromosomal level are responsible for the c-*sis* activation. Therefore altered regulation on the transcriptional level has to be considered.

Expression of the PDGF A-chain gene was found to be higher in malignant mesothelioma cell lines than in normal mesothelial cells. The three transcripts detected were the same as in placental RNA and as described by Betsholtz et al. (1986). In these cell lines, the number of copies of chromosome 7p corresponded to the ploidy

of the line which suggests that increased expression of PDGF A is probably a characteristic feature of malignant mesothelioma cell lines. The produced PDGF (A and/or B) could possibly function as an autocrine growth factor if the PDGF is secreted and if PDGF receptors are present on the malignant cell lines. Another possibility is the paracrine effect of the produced PDGF on connective tissue cells that results in the connective tissue growth which is frequently encountered in malignant mesotheliomas. Whether the expressed PDGF B- and PDGF A-chains in the malignant mesothelial cells are assembled as AB heterodimers or AA and BB homodimers and whether PDGF receptors occur on malignant mesothelioma cells remains to be established. Furthermore, it would be of great interest to study whether the expression of c-*sis* is also consistently enhanced in freshly obtained tumor material, because c-*sis* expression might become a useful marker for diagnosis of malignant mesotheliomas.

In summary, we have demonstrated enhanced expression of the c-*sis* oncogene in ten human malignant mesothelioma cell lines from primary and metastatic origin. This contrasts with the barely detectable level of c-*sis* expression in control mesothelial cells. Cytogenetic and restriction enzyme analysis revealed no indications for a mechanism responsible for the c-*sis* activation. These cell lines are extremely valuable for further investigations of the role of c-*sis* and PDGF A-chain expression in malignant transformation.

ACKNOWLEDGEMENTS

We gratefully acknowledge Prof.Dr. R. Benner for his continuous support. Dr. A. Geurts van Kessel, Dr. A. Hermans, Mr. S. van Baal, Ms. E. Schoenmaker, Ms. M. Moret and Ms I.L.M. Wolvers-Tettero are acknowledged for their advice and/or their technical assistance. Mr. C.J. van Dijk (Audiovisual Center of the Erasmus University) and Mr. T. van Os are acknowledged for their excellent photographic assistance. Dr. C. Betsholtz (University of Uppsala, Sweden) is acknowledged for generously providing the PDGF A-chain probe and Ms. J. de Goey-van Dooren for typing the manuscript. This study was supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

REFERENCES

Auffray, C. & Rougeon, F. (1980) Eur. J. Biochem., 107, 303-314.

Barret, T.B., Gajdusek, C.M., Schwartz, S.M., McDougall, J.K. & Benditt, E.P. (1984) Proc. Natl. Acad. Sci. USA, 81, 6772-6774.

Benham, F.J., Hodgkinson, S. & Davies, K.E. (1984) EMBO J, 3, 2635-2640.

Betsholtz, C., Heldin, C.-H., Nister, M., Ek, B., Wasteson, A. & Westermark, B. (1983) Biochem. Biophys. Res. Commun. 117, 176-182.

Betsholtz, C., Westermark, B., Ek, B., Heldin, C.-H. (1984) Cell, 39, 447-457.

Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L., Knott, T.J. & Scott, J. (1986) Nature, 320, 695-699.

Dalla Favera, R., Gallo, R.C., Giallongo, A. & Croce, C.M. (1982) Science, 218, 686-688.

Deuel, T.F., Huang, J.S., Huang, S.S., Stroobant, P. & Waterfield, M.D. (1983) Science, 221, 1348-1350.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. & Antoniades, H.N. (1983) Science, 221, 275-276.

Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C. & Aaronson, S.A. (1982) Nature, 295, 116-119.

Gerwin, B., Gabrielson, E., Lechner, J., Reddel, R., Roberts, A., Robbins, K. & Harris, C.C. (1987a) J. Cell Biochem., 11A, 58.
Gerwin, B.I., Lechner, J.F., Reddel, R., Betsholtz, C., Roberts, A. & Harris, C.C. (1987b) Eur. J. Cancer and Clin. Oncol., 23, 1752.
Goustin, A.S., Betsholtz, C., Pfeifer-Ohlsson, S., Persson H., Rydnert, J., Bywater, M., Holmgren, G., Heldin, C.-H., Westermark, B. & Ohlsson, R. (1985) Cell, 41, 301-312.
Graves, D.T., Owen, A.J., Barth, R.K., Tempst, P., Winoto, A., Fors, L., Hood, L.E. & Antoniades, H.N. (1984) Science, 226, 972-974.
Groffen, J., Heisterkamp, N., Stephenson, J.R., Geurts van Kessel, A., de Klein, A., Grosveld, G. & Bootsma, D. (1983) J. Exp. Med., 158, 9-15.
Heldin, C.-H. & Westermark, B. (1984) Cell, 37, 9-20.
Holley, R.W. (1975) Nature, 258, 487-490.
I.S.C.N. (1985) An International System for Human Cytogenetic Nomenclature Harnden, D.G. & Klinger, H.P. (eds) published in collaboration with Cytogenet. Cell Genet. Karger, Basel.
Jeffreys, A.J. &Flavell, R.A. (1977) Cell, 12, 429-439.
Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 202-204.
Martinet, Y., Bitterman, P.B., Mornex, J.-F., Grotendorst, G.R., Martin, G.R. & Crystal, R.G. (1986) Nature, 319, 158-160.
Morton, C.C., Bonthron, D.T. & Collins, T. (1987) HGM, 9, 98.
Nister, M., Heldin, C.-H., Wasteson, A. & Westermark, B. (1984) Proc. Natl. Acad. Sci. USA, 81, 926-930.
Pantazis, P., Pelicci, P.G., Dalla-Favera R. & Antoniades, H.N. (1985) Proc. Natl. Acad. Sci. USA, 82, 2404-2408.
Sporn, M.B. & Todaro, G.J. (1980) Engl. J. Med., 303, 878-880.
Swan, D.C., McBride, O.W., Robbins, K.C., Keithley, D.A., Reddy, E.P. & Aaronson, S.A. (1982) Proc. Natl. Acad. Sci. USA, 79, 4691-4695.
Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA, 77, 5201-5205.
Todaro, G.J. & De Larco, J.E. (1978) Cancer Res., 38, 4147-4154.
Wagner, J.C., Sleggs, C.A. & Marchand, P. (1960) Br. J. Ind. Med., 17, 260-270.
Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J.S. & Deuel, T.F. (1983) Nature, 304, 35-39.
Westermark, B., Johnsson, A., Paulsson, Y., Betsholtz, C., Heldin, C.-H., Herlyn, M., Rodeck, U. & Koprowski, H. (1986) Proc. Natl. Acad. Sci. USA, 83, 7197-7200.
Whitaker, D. and Shilkin, K.B. (1984) J. Pathol., 143, 147-175.

CHAPTER 7.2

EXPRESSION OF THE C-SIS ONCOGENE IN PLEURAL EFFUSION CELLS AND TUMOR MATERIAL OF HUMAN MALIGNANT MESOTHELIOMAS

ABSTRACT

Recently the expression of the c-*sis* oncogene was detected in human malignant mesothelioma cell lines. In the present study expression of the c-*sis* oncogene was demonstrated in total RNA isolated from freshly obtained material of eight malignant mesothelioma patients. The level of c-*sis* transcription varied between the different patients and did not correlate with the morphologically detected percentage tumor cells and/or macrophages, which can express the c-*sis* oncogene as well.

INTRODUCTION

Cellular proto-oncogenes are normal cellular genes with potential oncogenic activity (1,2). Several proto-oncogenes are known to be involved in normal proliferation and differentiation (3-5). This implies that aberrations in the expression of these proto-oncogenes or structural changes in their products may directly influence the growth of cells (6). Although the mechanism of activation is often unknown, expression of a number of oncogenes has been found in human tumors and data are accumulating about the relevance of this expression for diagnosis and prognosis. Monnat et al. (7) studied the expression of c-*myc*, c-H-*ras*, c-*fos* and c-K-*ras* in 17 patients with colon carcinoma and found a correlation between high levels of mRNA of these genes and biologically aggressive tumors. In human breast cancer expression of a *ras* protein has been found to be associated with the progression of the cancer (8).

The diagnosis of malignant mesothelioma is difficult as malignant mesothelioma cells cannot be easily distinguished from benign reactive mesothelial cells and adenocarcinoma cells (9). Recently, expression of the c-*sis* oncogene has been demonstrated in human malignant mesothelioma cell lines while cultured normal mesothelial cells did not show expression of this oncogene (10,11). The oncogene product $p28^{sis}$ is nearly identical to the B-chain of the platelet-derived growth factor (PDGF), which is a mitogen for connective tissue cells (12,13). We investigated whether c-*sis* expression is also detectable in freshly obtained tumor material of eight malignant mesothelioma patients. Total RNA was isolated from patient material and analysed by Northern blot.

MATERIALS AND METHODS

Patient material

Pleural effusions were collected in 50 ml tubes with 5000 IU heparin (Organon, Oss, The Netherlands) and 5 ml Ham's F10 medium (Gibco, Paisley, United Kingdom) supplemented with 15% fetal calf serum. After collection the material was immediately transported to the laboratory and the cells were harvested by centrifugation (10 min, 200 g). Subsequently, the pellets were quickly frozen in liquid nitrogen and stored at -70°C.

Autopsy was performed within 4 hours after death. Tumor material was frozen in liquid nitrogen immediately after autopsy and stored at -70°C.

Diagnosis

Diagnosis was established by routine cytology and always confirmed ultrastructurally and/or histologically (14).

RNA isolation and Northern blot analysis

RNA isolation and Northern blot analysis were performed as described (11). The probes used were a 1.7 BamHI c-*sis* fragment (15) and a 0.7 kb EcoRI-PstI GAPDH fragment (16).

TABLE I

Incidence of tumor cells and macrophages and expression of the c-*sis* oncogene in the collected patient material

patient	material	malignant mesothelioma cells[a] (%)	macrophages[a] (%)	c-*sis* transcription[b]
Me-25	pleural effusion	10	70	+
Me-62	pleural effusion	20	70	++
Me-67	pleural effusion	65	5	+
Me-83	pleural effusion	60	5	+
Me-84	pleural effusion	30	5	+
Me-85	pleural effusion	10	10	++
Me-102	pleural effusion	90	5	++
Me-77	tumor (autopsy)	-	-	+

a) Cytologically detected, b) Estimated from Fig. 1.

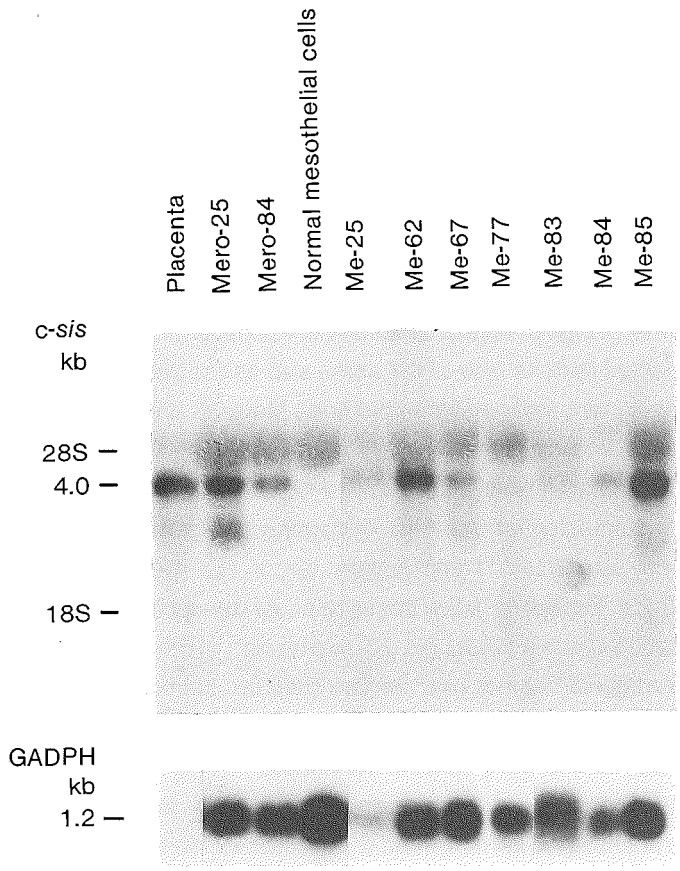

Figure 1. *Northern blot analysis of 20 µg total RNA of placenta, cultured normal mesothelial cells, two malignant mesothelioma cell lines (Mero-25 and Mero-84) and the patient material Me-25, -62, -67, -77, -83, -84 and -85. The blot was hybridized to ^{32}P-labeled c-sis* (top) and GAPDH probes (bottom).

RESULTS

Total RNA was isolated from tumor tissue from one and pleural effusion cells from eight malignant mesothelioma patients. Placental RNA and RNA from two malignant mesothelioma cell lines (Mero-25 and Mero-84) were used as positive controls and showed after hybridization with a c-*sis* probe a band of 4.0 kb (11). This band was lacking in the negative control of RNA isolated from cultured normal mesothelial cells. Figure 1 shows a Northern blot hybridized to the c-*sis* probe with the control RNA, and RNA from seven of the eight investigated patients Me-25, 62, 67, 77, 83, 84 and 85. The level of the detected c-*sis* transcription varied between the different patients. The quality and the amount of RNA applied were controlled by rehybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Figure 1).

Findings concerning the percentage of tumor cells and macrophages in the pleural effusions and a summary of the results from the Northern blot analysis are shown in table 1.

DISCUSSION

Expression of the c-*sis* oncogene was demonstrated in RNA isolated from tumor containing material of eight malignant mesothelioma patients. The band detected in the RNA from the patient material was of the same size as the bands in placental RNA and RNA from the malignant mesothelioma cell lines Mero-25 and Mero-84. The latter cell lines were isolated from the same patient material as the RNA samples Me-25 and Me-84, in which c-*sis* specific transcipts were detected as well (11).

Elevated expression of the c-*sis* oncogene has been reported for malignant mesothelioma cell lines (10,11) and several tumor cell lines isolated from different tumor types (18-20). Except for a few cases of acute myeloblastic leukemia (AML) and chronic myelocytic leukemia (CML) (21-23) expression of the c-*sis* oncogene in freshly obtained tumor material has not been reported so far. Slamon et al. (24) investigated twenty different tumors from 54 patients for expression of amongst others the c-*sis* oncogene but did not find c-*sis* specific transcripts.

The detection of c-*sis* expression in total RNA from patient material and especially pleural effusions is hampered by the low incidence of tumor cells and the presence of other cells e.g. macrophages which can express the c-*sis* proto-oncogene (25). The RNA *in situ* hybridization technique offers the possibility to distinguish c-*sis* positive cells with different morphology at the single cell level. This technique has been developed in our laboratory (26,27) but so far can only be applied to lymphocytes. Application on malignant mesothelioma cells in pleural effusions is hampered by high background and a weak signal. The latter could be due to low accessibility of the RNA.

Diagnosis of malignant mesothelioma on pleural effusions is often difficult due to lack of criteria to distinguish malignant mesothelioma cells from adenocarcinoma cells and reactive mesothelial cells (9). The detected c-*sis* expression by RNA *in situ* hybridization techniques may be useful for the diagnosis of malignant mesothelioma. The specificity of c-*sis* expression for malignant mesotheliomas is currently under investigation. Furthermore, it should be possible to enhance the differentiation between different c-*sis* positive cell types by application of a double staining technique, which combine membrane antigen labeling with the detection of RNA transcripts. Such procedures have to be developed and might be very useful in diagnostic procedures.

In summary, we have demonstrated that the expression of the c-*sis* oncogene, which has been detected in malignant mesothelioma cell lines, is also present in freshly obtained tumor material. Further investigations will reveal whether this observation is useful as a diagnostic tool and applicable in routine pathology.

REFERENCES

1. Cooper, G.M. Cellular transforming genes. Science 218, 801-806, 1982.
2. Bishop, J.M. Cellular oncogenes and retroviruses. Ann. Rev. Biochem. 52, 301-354, 1983.
3. Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wästeson, A., Westermark, B., Heldin, C.-H., Huang, J.S., Deuel, T.F. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304, 35-39, 1983.
4. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., Waterfield, M.D. Close similarity of epidermal growth factor receptor and v-*erbB* oncogene protein sequences. Nature 307, 521-527, 1984.
5. Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T., Stanley, E.R. The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41, 665-676, 1985.
6. Goustin, A.S., Leof, E.B., Shipley, G.D., Moses, H.L. Growth factors and cancer. Cancer Res. 46, 1015-1029, 1986.
7. Monnat, M., Tardy, S., Saraga, P., Diggelmann, H., Costa, J. Prognostic implications of expression of the cellular genes *myc*, *fos*, Ha-*ras* and Ki-*ras* in colon carcinoma. Int. J. Cancer 40, 293-299, 1987.
8. Clair, T., Miller, W.R., Cho-Chung, Y.S. Prognostic significance of the expression of a *ras* protein with a molecular weight of 21,000 by human breast cancer. Cancer Res. 47, 5290-5293, 1987.
9. Whitaker, D., Shilkin, K.B. Diagnosis of pleural malignant mesothelioma in life - a practical approach. J. Pathol. 143, 147-175, 1984.
10. Gerwin B.I., Lechner, J.F., Reddel, R.R., Roberts, A.B., Robbins, K.C., Gabrielson, E.W., Harris, C.C. Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47, 680-684, 1987.
11. Versnel, M.A., Hagemeijer, A., Bouts, M.J., van der Kwast, Th.H., Hoogsteden, H.C. Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene 2, 601-605, 1988.
12. Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J.S., Deuel, T.F. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of Simian sarcoma virus. Nature 304, 35-39, 1983.
13. Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., Antoniades, H.N. Simian sarcoma virus onc gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221, 275-277, 1983.
14. Van der Kwast, Th.H., Versnel, M.A., Delahaye, M., de Jong, A., Zondervan, P.E., Hoogsteden, H. Expression of epithelial membrane antigen on malignant mesothelioma cells. Acta Cytol. 32: 169-174, 1988.
15. Groffen, J., Heisterkamp, N., Stephenson, J.R., Geurts van Kessel, A., de Klein A., Grosveld, G. and Bootsma, D. c-*sis* is translocated from chromosome 22 to chromosome 9 in chronic myelocytic leukemia. J. Exp. Med. 158, 9-15, 1983.
16. Benham, F.J., Hodgkinson, S., Davies, K.E. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X chromosomes defines a multigene family. EMBO J. 3, 2635-2640, 1984.
17. Evinger-Hodges, M.J., Dicke, K.A., Gutterman, J.U., Blick, M. Proto-oncogene expression in human bone marrow. Leukemia 2, 597-602, 1987.
18. Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C., Aaronson, S.A. Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. Nature 295, 116-119, 1982.
19. Pantazis, P., Pelicci, P.G., Dalla-Favera, R., Antoniades, H.N. Synthesis and secretion of proteins resembling platelet-derived growth factor by human glioblastoma and fibrosarcoma cells in culture. Proc. Natl. Acad. Sci. USA 82, 2404-2408, 1985.
20. Graves, D.T., Owen A.J., Barth, R.K., Tempst, P., Winoto, A., Fors L., Hood, L.E., Antoniades, H.N. Detection of c-*sis* transcripts and synthesis of PDGF-like proteins by human osteosarcoma cells. Science 226, 972-974, 1984.

21. Evinger-Hodges, M.J., Bresser, J., Brouwer, R., Cox, I., Spitzer, G., Dicke, K. *myc* and *sis* expression in acute myelogenous leukemia. Leukemia 2, 45-49, 1988.
22. Sariban, E., Mitchell, T., Rambaldi, A., Kufe, D.W. c-*sis* but not c-*fos* gene expression is lineage specific in human myeloid cells. Blood 71, 488-493, 1988.
23. Romero, P., Blick, M., Talpaz, M., Murphy, E., Hester, J., Gutterman, J. c-*sis* and c-*abl* expression in chronic myelogenous leukemia and other hematologic malignancies. Blood 67, 839-841, 1986.
24. Slamon, D.J., deKernion, J.B., Verma, I.M., Cline, M.J. Expression of cellular oncogenes in human malignancies. Science 224, 256-262, 1984.
25. Martinet, Y., Bitterman, P.B., Mornex, J.-F., Grotendorst, G.R., Martin, G.R., Crystal, R.G. Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity. Nature 319, 158-160, 1986.
26. Bakkus, M.H.C., Versnel, M.A., Adriaansen, H.J., Van den Akker, Th.W. Non-isotopic RNA *in situ* hybridization: The detection of low copy number mRNA molecules in bone marrow cells of multiple myeloma patients. In: Monoclonal Gammapathies II: Clinical significance and basic mechanisms. Radl, J. van Van Camp, B. (Eds.) 1989, pp 113-116.
27. Bakkus, M.H.C. Brakel-van Peer, K.M.J., Adriaansen, H.J., Wierenga-Wolf, A.F., van den Akker, Th.W., Dicke-Evinger, M.J., Benner, R. Detection of oncogene expression by fluorescent *in situ* hybridization in combination with immunofluorescent staining of cell surface markers. Oncogene, in press.

CHAPTER 7.3

EXPRESSION OF PDGF RECEPTORS IN HUMAN MALIGNANT MESOTHELIOMA CELL LINES AND CULTURED NORMAL MESOTHELIAL CELLS

M.A. Versnel[1], L. Claesson-Welsh[4], A. Hammacher[4], M.J. Bouts[1], Th.H. van der Kwast[2], R. Willemsen[1], S.M. Weima[5], H.C. Hoogsteden[3], A. Hagemeijer[1] and C.-H. Heldin[4]

[1]Department of Cell Biology, Immunology and Genetics, [2]Department of Pathology and [3]Department of Pulmonary Medicine, Erasmus University and Academic Hospital Dijkzigt, Rotterdam, The Netherlands, [4]Ludwig Institute for Cancer Research, Uppsala, Sweden and [5]Hubrecht Laboratory, Utrecht, The Netherlands

A modified version of this chapter will be published.

ABSTRACT

In human malignant mesothelioma cell lines elevated expression of the PDGF B-chain (c-*sis*) gene was previously reported, while normal mesothelial cells barely express this gene. Expression of the PDGF A-chain gene was only slightly elevated in these cell lines compared to normal mesothelial cells. For an autocrine function of the produced PDGF in these cells expression of PDGF receptors is a prerequisite. In this paper we report on the inversed pattern of expression of PDGF A- and B-type receptors in normal and malignant mesothelial cells. Cultured normal mesothelial cells expressed the PDGF A-type receptor mRNA and had weak to undetectable levels of the PDGF B-type receptor mRNA and protein. In contrast, malignant mesothelioma cell lines were found to express PDGF B-type receptor mRNA and protein, while the PDGF A-type receptor mRNA was hardly detectable. The presence of plasma membrane associated B-type receptors was demonstrated by binding experiments with ^{125}I-PDGF-BB. Nevertheless, immunoelectron microscopy revealed that the majority of the PDGF receptors was found in lysosomes and presumptive vesicles in the cytoplasm. In summary, these results indicate that all components for a PDGF-dependent growth stimulation of malignant mesothelioma cells are present.

INTRODUCTION

The platelet-derived growth factor (PDGF) was initially isolated from human platelets and identified as a dimeric molecule consisting of A- and B-polypeptide chains (Johnsson et al., 1982; Ross et al., 1986). The PDGF B-chain is nearly identical to the protein encoded by the v-*sis* oncogene (Doolittle et al., 1983; Van den Ouweland et al., 1985; Waterfield et al., 1983). The PDGF A-chain gene is 60% similar to the B-chain (Betsholtz et al., 1986). Expression of a single PDGF gene or both genes has been found in a variety of normal and tumor cell lines (Barret et al., 1984; Eva et al., 1982; Martinet et al., 1986; Westermark et al., 1986). All possible dimeric combinations of A and B chains (AA, AB and BB) have been identified in normal and transformed cells (Heldin et al., 1986; Hammacher et al., 1988; Stroobant and Waterfield, 1984). The oncogenic effect of c-*sis* has been attributed to an autocrine growth stimulating activity, which requires that the cells producing c-*sis* also have functional receptors for the produced factor (Todaro and De Larco, 1978; Sporn and Todaro, 1980; Heldin and Westermark, 1984).

Binding experiments with the different isoforms of PDGF to cultured cells revealed the existence of two distinct receptor types, denoted type-A and type-B

Heldin et al., 1988; Hart et al., 1988). The PDGF B-type receptor binds PDGF-BB with high affinity, and with lower affinity PDGF-AB, while it does not bind PDGF-AA. The A-type receptor binds all three dimeric forms with high affinities. The human PDGF A- and B-type receptor were recently cloned and were found to be structurally related (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988, Matsui et al., 1989; Claesson-Welsh et al., 1989b).

Malignant mesotheliomas are mesodermally derived tumors. Human malignant mesothelioma cell lines have been found to express both PDGF A- and B-chain genes (Gerwin et al., 1987; Versnel et al., 1988). In contrast, normal mesothelial cell cultures were found to express the A-chain gene while B-chain transcripts were barely detectable (Versnel et al., 1988). Here, we report that whereas cultured normal mesothelial cells express the PDGF A-type receptor and weak levels of the B-type receptor, the reverse is true for mesothelioma cell lines that generally express the B-type receptor, but have a weak to undetectable level of the A-type receptor.

MATERIALS AND METHODS

Cell lines and growth conditions

The malignant mesothelioma cell lines and normal mesothelial cell cultures were isolated and cultured as described (Versnel et al., 1989).

RNA isolation and Northern blot analysis

RNA isolation and Northern blot analysis were performed as described (Versnel et al., 1988). The filters were washed at 42°C to 0.3xSSC and exposed to a Fuji-RX film.

Probes

The PDGF B-type receptor probe was a 1 kb *Pst* fragment derived from cDNA clone HPDGFR-2A3 (Claesson-Welsh et al., 1988) and the PDGF A-type receptor probe was a 1.5 kb *Eco*RI fragment (Claesson-Welsh et al., 1989). The fragments correspond to the extracellular domain of the B- and A-type receptors, respectively. The GAPDH probe was a 0.7 kb *Eco*RI-*Pst*I fragment (Benham et al., 1984).

PDGF and radiolabeled ligands

PDGF-AA and PDGF-BB were recombinant material produced in yeast cells (Östman et al., 1989) and ^{125}I-labeled by the method of Bolton and Hunter (1972) to specific activities of 55,000 and 80,000 cpm/μg, respectively.

Radioreceptor assay

Cells were plated in 12-well plates (Costar, Cambridge, MA). When confluence was reached, the cells were washed with phosphate buffered saline (PBS) and incubated for 24 hours in serum free F10 medium at 37°C. Prior to binding, the cells were washed with 1 ml PBS supplemented with bovine serum albumin (BSA), 1 mg/ml (Boehringer, Mannheim, F.R.G.). Binding was performed for two hours at 0°C in a volume of 350 μl PBS-BSA containing radiolabeled ligands with or without unlabeled PDGF (250 ng per well). After five washes with ice cold PBS-BSA the cells were lysed in 2% Triton X-100 in H_2O. ^{125}I-radioactivity was determined in a gamma counter.

Metabolic labeling and immunoprecipitation

Cells were cultured as described above. After washing twice with PBS, the cells were incubated in methionine-free DMEM (Flow Laboratories, Irvine, United Kingdom). After one hour, the medium was changed to 10 ml methionine-free medium containing 2% dialyzed fetal calf serum, and 50 μCi ^{35}S-methionine (Amersham International, Amersham, U.K.). After 6 hours labeling the cells were lysed as described (Claesson-Welsh et al., 1987). The lysate was centrifuged for 0.5 min at 10,000 g. The supernatant was precleared by overnight incubation at 4°C with normal rabbit serum and protein A-Sepharose (Pharmacia, Uppsala, Sweden) and then incubated overnight at 4°C with the rabbit antiserum PDGFR-3, which specifically recognizes the PDGF B-type receptor (Claesson-Welsh et al., 1989b). Further procedures were performed as described (Claesson-Welsh et al., 1987). Immunoprecipitates were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis according to Laemmli (1970) using 6% polyacrylamide gels.

Immunocytochemistry

For immunoelectron microscopy we used cultured cells as described above and tumor cells from the pleural fluid of a patient with a well-established diagnosis of malignant mesothelioma. Fixation and embedding procedures for ultracryotomy were performed as described before (Willemsen et al., 1988). The antiserum used was the anti-PDGF B-type receptor antibody PDGFR-HL2 that recognizes the intracellular part of the B-type receptor (Weima et al., submitted). PDGF B-type receptors were visualized by the indirect immunogold method using gold probes as marker (Willemsen et al., 1988). As a control for specificity sections were incubated with normal rabbit serum instead of the primary antibody. The observed background labeling was negligible.

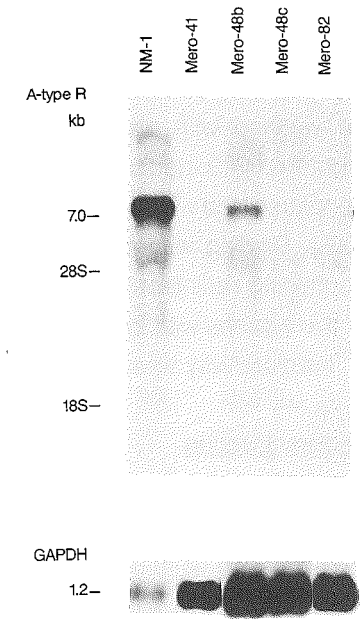

Figure 1. Northern blot analysis of 5 µg poly(A)$^+$ RNA from placenta, normal mesothelial cells and malignant mesothelioma cell lines hybridized to a [^{32}P]-labeled PDGF A-type receptor probe and a GAPDH probe.

RESULTS

Expression of the PDGF A- and B-type receptor mRNA molecules in normal and malignant mesothelial cell lines

RNA was isolated from human malignant mesothelioma cell lines and normal mesothelial cell cultures. The 7 kb PDGF A-type receptor mRNA was clearly detectable in poly(A)$^+$ RNA from normal mesothelial cells, while malignant mesothelioma cell lines showed even after long exposure a weak to undetectable expression (Figure 1). For the PDGF B-type receptor, placental RNA was used as a positive control and showed a 6 kb mRNA molecule. All twelve investigated malignant mesothelioma cell lines except Mero-95 expressed the 6 kb PDGF B-type receptor specific mRNA (Figures 2A and 2B). The level of PDGF B-type receptor expression varied between the different cell lines: strong expression in Mero-14, -72, -96, -48a, -48b, -48c and -48d, intermediate expression in Mero-41 and -82, and weak expression in Mero-25 and -84. The normal mesothelial cell cultures showed a weak expression of the PDGF

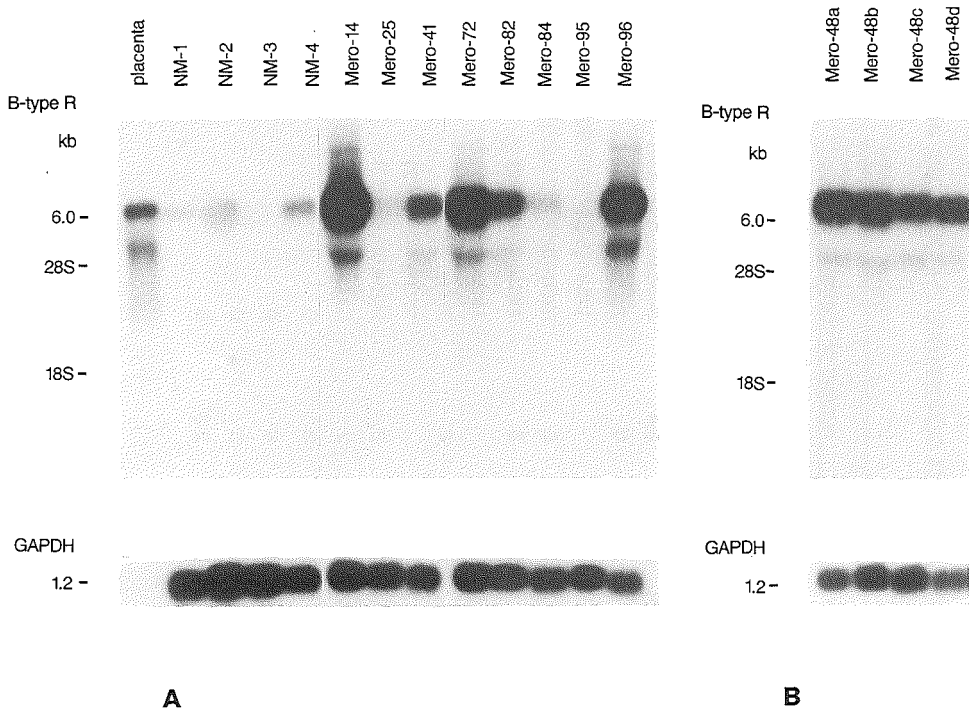

Figure 2. Northern blot analysis of 20 µg total RNA from placenta, normal mesothelial cells (NM1-4) and 12 malignant mesothelioma cell lines hybridized to a [^{32}P]-labeled PDGF B-type receptor probe and a GAPDH probe.

B-type receptor in three out of four cultures tested (Figure 2A). The amount of RNA applied and the quality were controlled by rehybridization of the filters with a GAPDH probe. In placental RNA GAPDH transcripts were only detectable after long exposure. Variable expression of the PDGF B-chain in this panel of malignant mesothelioma cell lines was reported previously (Versnel et al., 1988). In five cell lines an inverse relationship was observed between levels of expression of PDGF B-type receptor and PDGF B-chain mRNA: the malignant mesothelioma cell lines Mero-41 and Mero-82 had a high c-*sis* expression and a weak PDGF B-type receptor expression whereas Mero-14, Mero-72 and Mero-96, which had a weak c-*sis* expression, were found to have a strong PDGF B-type receptor expression. In the malignant mesothelioma cell lines derived from a primary tumor (Mero-48a) and its metastases (Mero-48b, c, d) the expression of the c-*sis* and the B-type receptor was intermediate. A summary of these data is shown in Table 1.

TABLE I

SUMMARY OF DATA ON PDGF AND PDGF RECEPTOR EXPRESSION, AND CYTOGENETIC DATA CONCERNING CHROMOSOME #4 and #5 IN MALIGNANT MESOTHELIOMA CELL LINES

	PDGF A*	PDGF B*	PDGF A-type receptor		PDGF B-type receptor			modal chromosome number	number of copies		
	mRNA	(c-*sis*) mRNA	mRNA	RRA**	mRNA	Immunoprecipitation	RRA		normal 4	normal 5	rearranged 5
Mero-14	+	+	-	+	+++	ND	+	75(72-78)	2	4	0
Mero-25	++	+	-	-	±	+	+	135(127-140)	3-4	3-4	3-4xdel(5)(q21q34)
Mero-41	+++	+++	-	-	+	ND	+	72(68-75)	2	0	2xinv(5)(p15q11) 1x5p+[5qter→p15::6q15ter]
Mero-48a	++	++	-	ND	++	ND	ND	71-75	2*	2	2x5q-[del(5)(q11q22)]
Mero-48b	++	++	-	±	++	+	+	71-75	2*	2	1xdel(5)(q11q22) 1xdel(5)(q11q22)q+
Mero-48c	++	++	±	+	++	ND	+	71-75	2*	2	1x5q+(HSR) 1xdel(5)(q11q22)
Mero-48d	++	++	-	ND	++	ND	ND	71-75	2*	2	2xdel(5)(q11q22)
Mero-72	+	++	-	ND	+++	ND	ND	41-43	1	2	0
Mero-82	+++	++	-	±	+	+	+	48-50	2	3	0
Mero-84	++	+	ND	-	±	ND	+	38	1	2	0
Mero-95	+	++	ND	-	-	+	±	54-58	2	3	0
Mero-96	+	++	ND	ND	+++	+	ND	72-78	3	2-3	0
normal mesothelial cells	+	-	+	ND	-/±/+	+	ND				

*Versnel et al., 1988, **Radioreceptor assay, ND=not done.

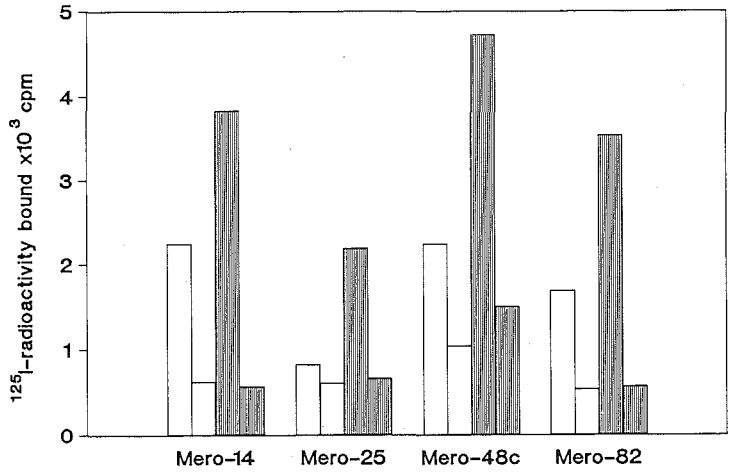

Figure. 3 Binding of ^{125}I-PDGF-AA and ^{125}I-PDGF-BB by 4 human malignant mesothelioma cell lines.

The PDGF A-type receptor has been mapped to chromosome 4 (Matsui et al., 1989; Stenman et al., 1989). Loss of one copy of chromosome 4 is frequently observed in malignant mesothelioma cell lines. However, this cannot explain the absence of PDGF A-type mRNA expression as loss of chromosome 4 is not a consistent feature of all the investigated cell lines (Table 1).

The PDGF B-type receptor has been mapped to chromosome 5 (Yarden et al., 1986). The level of expression in the malignant mesothelioma cell lines does not correlate with the number of copies of chromosome 5 or with a specific marker for chromosome 5 (Table 1).

Radioreceptor assay with ^{125}I-PDGF-AA and ^{125}I-PDGF-BB.

To investigate whether PDGF A-type and PDGF B-type receptors were present on the malignant mesothelioma cell lines, binding experiments with ^{125}I-PDGF-AA and ^{125}I-PDGF-BB were performed. For competition an excess of unlabeled PDGF-AA and PDGF-BB was used. Eight malignant mesothelioma cell lines were investigated and were found to bind ^{125}I-PDGF-BB (Figure 3 and table 1). The binding of iodinated ligand was competed for by incubation with an excess of unlabeled PDGF-AA or PDGF-BB. Mero-95, which did not express detectable levels of PDGF B-type receptor mRNA, had only a low binding of ^{125}I-PDGF-BB. The malignant mesothelioma cell lines

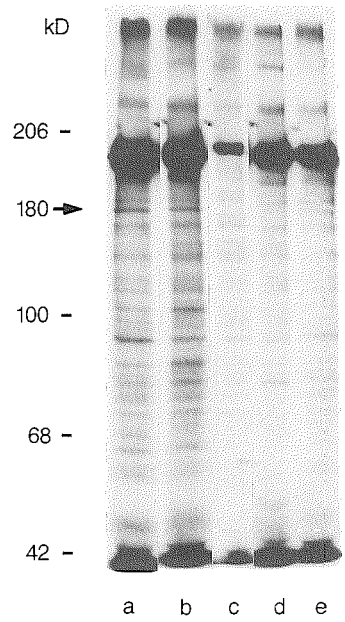

Figure 4. *Immunoprecipitation using an antiserum reactive with the PDGF B-type receptor from metabolically labeled malignant mesothelioma cell lines (lane a: Mero-25, lane b: Mero-82, lane d: normal mesothelial cells NM-4, lane e: normal mesothelial cells NM-1. Lane c shows the immunoprecipitation using an irrelevant antiserum and metabolically labeled Mero-82 cells.*

Mero-14, Mero-48c and Mero-82 displayed a low specific binding of ^{125}I-PDGF-AA (Figure 3). In the other investigated malignant mesothelioma cell lines the binding was not significantly over the background (Table 1). Normal mesothelial cells could not be tested with this assay as these cells could not resist the intensive washing procedures.

Immunoprecipitation

The presence of PDGF B-type receptors in normal and malignant mesothelial cells was also demonstrated by immunoprecipitation. For the detection of low numbers PDGF receptors the cell lines Mero-25, Mero-48b, Mero-82 and Mero-95 and two normal mesothelial cell lines were metabolically labeled with ^{35}S-methionine and subjected to immunoprecipitation with an antiserum reactive with the PDGF B-type receptor. SDS gel electrophoresis revealed bands of approximately 180 kd in the malignant mesothelioma cell lines as well as in the normal mesothelial cell cultures, i.e.

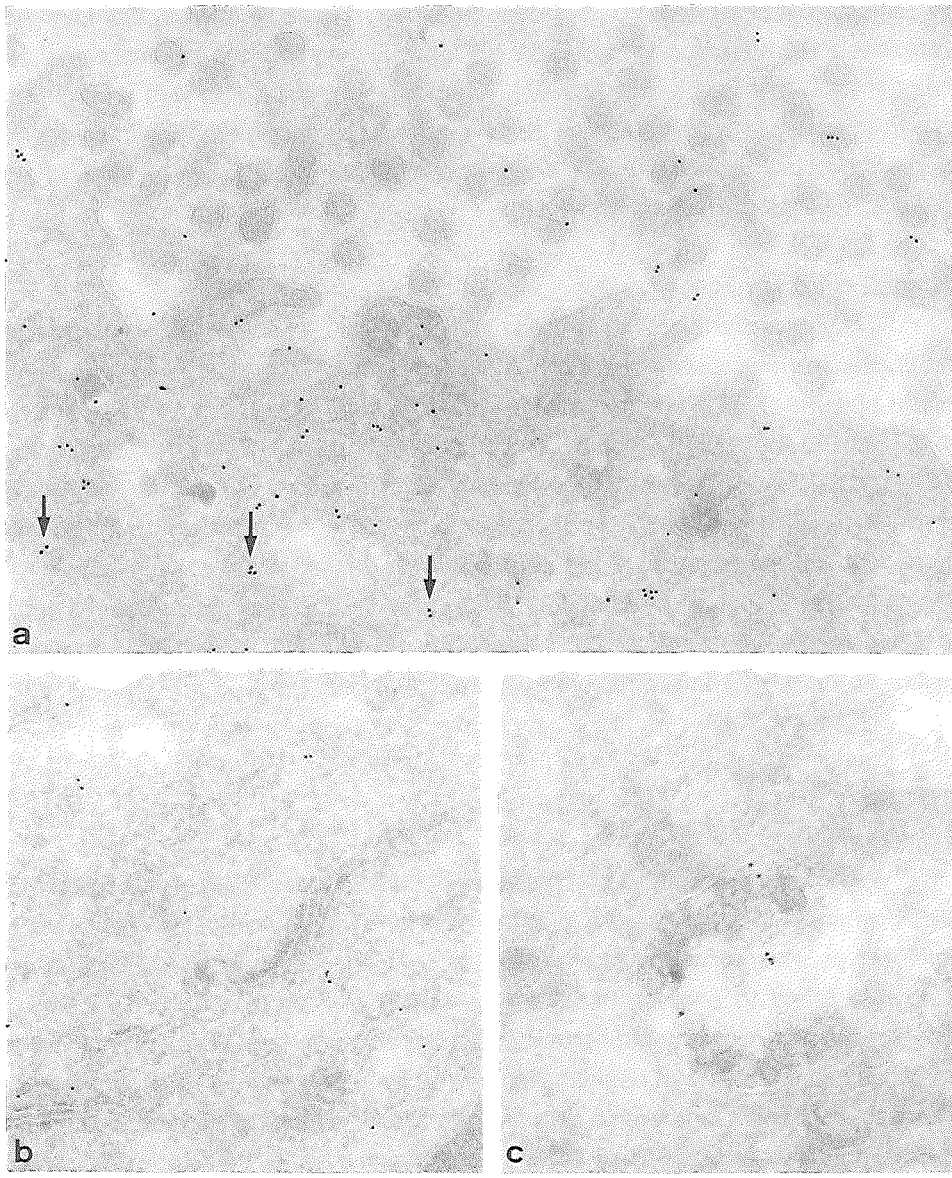

Figure 5 *a. Subcellular distribution of PDGF B-type receptor in malignant mesothelioma cells of a patient using specific antiserum. The antigen is found on the plasma membrane and often in small vesicular structures (arrows). b. Detail of the rough endoplasmic reticulum of Mero-14 cells showing labeling for PDGF B-type receptor. c. Higher magnification showing a lysosomal structure in Mero-14 cells with weak labeling for PDGF B-type receptor.*

the expected size of the PDGF B-type receptor. Figure 4 shows the immunoprecipitated 180 kd molecule from Mero-25, Mero-82 and two normal mesothelial cell lines (lanes a, b, d and e, respectively). After immunoprecipitation with an irrelevant antiserum the 180 kd band was not observed (lane c). From Mero-95, which did not express detectable levels of PDGF B-type receptor mRNA, only a weak band of 180 kd was precipitated (data not shown).

Immunoelectron microscopy

The subcellular localization of the PDGF B-type receptor was investigated by immunoelectron microscopy of ultrathin frozen sections of malignant mesothelioma cells in a pleural effusion (Figure 5A) and of the malignant mesothelioma cell line Mero-14 (Figure 5B and C). The distribution of the gold particles observed in the patient material and the cultured malignant mesothelioma cell lines was similar. Figure 5A shows labeling for the PDGF B-type receptor in the patients tumor cells on the plasma membrane with preference for the microvilli. Also a labeling pattern just below the plasma membrane often associated with small presumptive vesicular structures could be detected. Furthermore labeling was found of the rough endoplasmic reticulum (Figure 5B) and lysosomal structures (Figure 5C).

DISCUSSION

We report here that normal mesothelial cells express PDGF A-type receptor mRNA, whereas these cells were found to have only weak or undetectable levels of PDGF B-type receptor mRNA and protein. In contrast, malignant mesothelioma cell lines were found to express PDGF B-type mRNA and protein, but only weak or undetectable levels of A-type mRNA. Since normal mesothelial cells are cultured in medium supplemented with epidermal growth factor (EGF) and hydrocortisone (HC), whereas malignant mesothelioma cells are cultured without these supplements, it was possible that the expression of PDGF receptors in normal mesothelial cells was influenced by these supplements. However, addition of EGF and HC to malignant mesothelioma cell lines did not change the expression of PDGF A-type receptors, as determined by binding experiments and Northern blotting, making this possibility unlikely (data not shown).

Recently we described clear but variable expression of the c-*sis* (PDGF B) oncogene in ten malignant mesothelioma cell lines while c-*sis* was barely detectable in normal mesothelial cells (Versnel et al., 1988). Expression of the PDGF A-chain was found to be slightly enhanced in the malignant mesothelioma cell lines compared to

normal mesothelial cells. These results taken together with the demonstration of PDGF B-type receptors on normal and malignant mesothelial cells suggest that PDGF-BB produced by the malignant mesothelioma cells could function as an autocrine growth factor and play a role in the stimulation of growth of the tumor cells. Since normal mesothelial cells express both PDGF A-chain and A-type receptor mRNA, it is possible that PDGF-AA is involved in autocrine stimulation of normal mesothelial cells.

Recently, Huang and Huang (1988) reported that in v-*sis* or c-*sis* transformed cells the majority of the newly synthesized PDGF receptors did not reach the cell membrane and that these receptors were rapidly degraded. According to this observation a cytoplasmic interaction between the c-*sis* gene product and its receptor was suggested. The presence of membrane associated PDGF receptors on the malignant mesothelioma cell lines was investigated by binding experiments with ^{125}I-PDGF-AA and ^{125}I-PDGF-BB. The specific binding of ^{125}I-PDGF-AA to the different malignant mesothelioma cell lines was low or around detection level. This observation is consistent with the lack of clearly detectable PDGF A-type mRNA expression. All malignant mesothelioma cell lines investigated were found to bind ^{125}I-PDGF-BB, which indicates towards the presence of plasma membrane associated PDGF B-type receptors. Immunoelectron microscopical studies of PDGF B-type receptors in malignant mesothelioma cell lines revealed that although plasma membrane associated PDGF B-type receptors occur, most receptors are found in lysosomes and presumptive vesicles in the cytoplasm. This observation might indicate towards an intracellular degradation of PDGF B-type receptors and a possible intracellular interaction between PDGF and its receptor.

It was reported that SSV-transformed mouse fibroblasts and normal rat kidney cells secreted PDGF while no PDGF was detected in the conditioned medium of SSV-transformed monkey fibroblasts (Huang et al., 1984). The detected amounts of secreted PDGF were found to correlate with the rate of tumor growth after injection into nude mice. The failure of SSV-transformed monkey fibroblasts to secrete detectable levels of PDGF suggests an intracellular interaction of PDGF with its receptor. The malignant mesothelioma cell lines investigated so far were also found to secrete minor amounts of PDGF protein and attempts to grow malignant mesothelioma cell lines in nude mice resulted only once in a tumor (M.A. Versnel, unpublished results). These observations are consistent with an intracellular interaction.

In summary, we have demonstrated expression of the PDGF B-type receptor in a panel of human malignant mesothelioma cell lines, which were previously reported to express PDGF B- and PDGF A-chain genes. Cultured normal mesothelial cells were found to express PDGF A-type receptors while malignant mesothelioma cell lines had a weak to undetectable expression of this gene. These results indicate that all

components for a PDGF-dependent autocrine growth stimulation of malignant mesothelioma cells are present. Furthermore, malignant and normal mesothelial cells could be a useful model for the study of mechanism involved in the regulation of expression of PDGF A- and B-type receptors.

ACKNOWLEDGEMENTS

We gratefully acknowledge Prof. Dr. R. Benner for his continuous support. Dr. A.C. van Denderen, Dr. A. Hoogeveen, Ms. E. van Drunen, Ms. M. Moret and Ms. E. Franken-Postma are acknowledged for their advice and/or technical assistance. Mr. T. van Os is acknowledged for photographic assistance and Ms. J. de Goey-van Dooren and G. de Korte for typing the manuscript. This study was supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

REFERENCES

Barrett, T.B., Gajdusek, C.M., Schwartz, S.M., McDougall, J.K., Benditt, E.P. (1984). Expression of the *sis* gene by endothelial cells in culture and *in vivo*. Proc. Natl. Acad. Sci. USA 81, 6772-6774.

Benham, F.J., Hodgkinson, S., Davies, K.E. (1984). A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X chromosomes defines a multigene family. EMBO J. 3, 2635-2640.

Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L., Knott, T.J., Scott, J. (1986). cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. Nature 320, 695-699.

Bolton, A.E. and Hunter, W.M. (1983). The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 133, 529-539.

Claesson-Welsh, L., Rönnstrand, L., Heldin, C.-H. (1987). Biosynthesis and intracellular transport of the receptor for platelet-derived growth factor. Proc. Natl. Acad. Sci. USA 84, 8796-8800.

Claesson-Welsh, L., Eriksson, A., Moren, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C., Heldin, C.-H. (1988) cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. Molec. Cell. Biol. 8, 3476-3486.

Claesson-Welsh, L., Hammacher, A., Westermark, B., Heldin, C.-H. Nistér, M. (1989a). Identification and structural analysis of the A-type receptor for PDGF, Similarities with the B-type receptor. J. Biol. Chem. 264, 1742-1747.

Claesson-Welsh, L., Eriksson, A., Westermark, B., Heldin, C.-H. (1989b). cDNA cloning and expression of the human A type PDGF receptor establishes structural similarity to the B type receptor. Proc. Natl. Acad. Sci. USA, in press.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., Antoniades, H.N. (1983). Simian sarcoma virus onc gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221, 275-277.

Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C., Aaronson, S.A. (1982). Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. Nature 295, 116-119.

Gerwin, B.I., Lechner, J.F., Reddel, R.R., Roberts, A.B., Robbins, K.C., Gabrielson, E.W., Harris, C.C. (1987). Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47, 6180-6184.

Gronwald, R.G.K., Grant, F.J., Haldeman, B.A., Hart, C.E., O'Hara, P.J., Hagen, F.S., Ross, R., Bowen-Pope, D.F., Murray, M.J. (1988). Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: Evidence for more than one receptor class. Proc. Natl. Acad. Sci. USA 85, 3435-3439.

Hammacher, A., Hellman, U., Johnsson, A., Östman A., Gunnarsson, K., Westermark, B., Wästeson, A., Heldin, C.-H. (1988). A major part of plateled-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. J. Biol. Chem. 263, 16493-16498.

Hart, C.E., Forstrom, J.W., Kelly, J.D., Seifert, R.A., Smith, R.A., Ross, R., Murray, M.J. and Bowen-Pope, D.F. (1988). Two classes of PDGF receptor recognize different isoforms of PDGF. Science 240, 1529-1531.

Heldin, C.-H., Westermark, B. (1984). Growth factors: Mechanism of action and relation to oncogenes.

Cell 37, 9-20.
Heldin, C.-H., Johnsson, A., Ek, B., Wennergren, S., Rönnstrand, L., Hammacher, A., Faulders, B., Wästeson, A., Westermark, B. (1987). Purification of human platelet-derived growth factor. Meth. Enzymol. 147, 3-13.
Heldin, C.-H., Johnsson, A., Wennergren, S., Wernstedt, C., Betsholtz, C., Westermark, B. (1986). A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Nature 319, 511-514.
Heldin, C.-H., Bäckstrom, G., Östman, A., Hammacher, A., Rönnstrand, L., Rubin, K., Nistér, M., Westermark, B. (1988).Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J. 7, 1387-1393.
Huang, J.S., Huang, S.S., Deuel, T.F. (1984). Transforming protein of Simian sarcoma virus stimulates autocrine growth of SSV-transformed cells though PDGF cell-surface receptors. Cell 39, 78-87.
Huang, S.S., Huang, J.S. (1988). Rapid turnover of the platelet-derived growth factor receptor in *sis*-transformed cells and reversal by suramin. J. Biol. Chem. 263, 12608-12618.
Johnsson, A., Heldin, C.-H., Westermark, B., Wästeson, A. (1982). Platelet-derived growth factor: Identification of constituent polypeptide chains. Biochem. Biophys. Res. Commun. 104, 66-74.
Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
Martinet, Y., Bitterman, P.B., Mornex, J.-F., Grotendorst, G.R., Martin, G.R., Crystal, R.G. (1986). Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity. Nature 319, 158-160.
Matsui, T., Heidaran, M., Mibi, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J. and Aaronson, S. (1989). Isolation of a novel receptor cDNA established the existence of two PDGF receptor genes. Science 243, 800-803.
Östman, A., Backstrom, G., Fong, N., Betsholtz, C., Wernstedt, C., Hellman, U., Westermark, B., Valenzuela, P. and Heldin C.-H. (1989). Expression of three recombinant homodimeric isoforms of PDGF in Saccharomyces cerevisiae. Growth Factors, in press.
Peres, R., Betsholtz, C., Westermark, B., Heldin, C.-H. (1987). Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. Cancer Res. 47, 3425-3429.
Ross, R., Raines, E.W. and Bowen Pope, D.F. (1986). The biology of the platelet-derived growth factor. Cell 46, 155-169.
Sitaras, N.M., Sariban, E., Bravo, M., Pantazis, P. and Antoniades, H.N. (1988). Constitutive production of platelet-derived growth factor-like proteins by human prostate carcinoma cell lines. Cancer Res. 48, 1930-1935.
Sporn, M.B., Todaro, G.J. (1980). Autocrine secretion and malignant transformation of cells. New Engl. J. Med. 303, 878-880.
Stenman, G., Eriksson, A. and Claesson-Welsh, C. (1989). The human PDGF A-type receptor gene maps to the same region on chromosome 4 as the KIT proto-oncogene. Genes, Chromosomes and Cancer, in press.
Stroobant, P. and Waterfield, M.d. (1984). Purification and properties of porcine platelet-derived growth factor. EMBO J. 12, 2963-2967.
Todaro, G.J., De Larco, J.E. (1978). Growth factors produced by sarcoma virus-transformed cells. Cancer Res. 38, 4147-4154.
Van den Ouweland, A.M.W., Breuer, M.L., Steenbergh, P.H., Schalken, J.A., Bloemers, H.P.J., Van de Ven, W.J.M. (1985) Comparative analysis of the human and feline c-*sis* proto-oncogenes. Identification of 5' human c-*sis* coding sequences that are not homologous to the transforming gene of simian sarcoma virus. Biochim. Biophys. Acta 825, 140-147.
Versnel, M.A., Hagemeijer, A., Bouts, M.J., Van der Kwast, Th.H., Hoogsteden, H.C. (1988) Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene 2, 601-605.
Versnel, M.A., Bouts, M.J., Hoogsteden, H.C., van der Kwast, Th.H., Delahaye, M. and Hagemeijer, A. Establishment of human malignant mesothelioma cell lines. Int. J. Cancer, in press.
Yarden, Y., Escobedo, J.A., Kuang, W.-J. Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E., Harkins, R.N., Francke, U., Fried, V.A., Ullrich, A., Williams, L.T. (1986) Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature 323, 226-232.
Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wästeson, A., Westermark, B., Heldin, C.-H. Huang, J.S., Deuel, T.F. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304, 35-39.
Westermark, B., Johnsson, A., Paulsson, Y., Betsholtz, C., Heldin, C.-H., Herlyn, M., Rodeck, U., Koprowski, H. (1986). Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor. Proc. Natl. Acad. Sci. USA 83, 7197-7200.
Weima, S.M., Van Rooyen, M.A., Mummery, C.L., Feyen, A., de Laat, S.W. and van Zoelen, E.J.J.

Identification of the type B-receptor for Platelet Derived Growth Factor in human embryonal carcinoma cells. Submitted.
Willemsen, R., van Dongen, J.M., Aerts, J.M.F.G., Schram, A.W., Tager, J.M., Goudsmit, R. and Reuser, A.J.J. (1988). An immunoelectron microscopic study of glucocerebrosidase in Type 1 Gau disease in spleen. Ultrastruct. Path. 12, 471-478.

CHAPTER 8

GENERAL DISCUSSION

Diagnosis of human malignant mesothelioma is difficult as malignant mesothelioma cells lack specific characteristics. The major problem in diagnosis of malignant mesothelioma by cytology is to distinguish malignant from benign mesothelial cells and malignant mesothelial cells from adenocarcinoma. The purpose of this study was to improve the diagnosis of human malignant mesothelioma and to increase our insight into the growth regulation and transformation of normal mesothelial cells.

8.1 Diagnostic methods for malignant mesothelioma

Most patients with malignant mesothelioma have a pleural effusion at initial presentation. Diagnosis of malignant mesothelioma is limited by the difficulty of distinguishing malignant mesothelioma cells from benign mesothelial cells and adenocarcinoma by routine cytology. A reliable diagnosis on pleural effusions is important as it prevents more invasive diagnostic procedures like thoracoscopies or percutaneous biopsies. In this thesis improvement of diagnosis was attempted by the study of cytogenetic aberrations and by immunocytochemical and immunoelectron microscopical investigations.

Several groups have studied the expression of epithelial membrane antigen (EMA) in malignant mesothelioma but their results are controversial. Malignant mesotheliomas were found negative for EMA by Battifora et al. (1984) while others found malignant mesotheliomas positive for EMA (Marshall et al., 1984; Pinkus et al., 1985; Sloane et al., 1983). In section 5.1, immunocytochemistry and immunoelectron microscopy were used for the study of the expression of EMA on pleural effusion cells of 25 patients with a malignant mesothelioma. EMA-positive cells were detected in 23 samples. In three cases of the 56 investigated cytologically negative control smears, EMA-positive cells were detected. One month later the pleural effusion of one of these patients was found to contain numerous malignant epithelial cells. The significance of the EMA-positive cells in the other two cases is unknown but other investigators also found occasionally EMA-positive cells in benign effusions (To et al., 1981; Gosh et al., 1983). In the other remaining 53 smears the reactive mesothelial cells were found to be EMA-negative. The conclusion that can be drawn from this study is that malignant mesothelial cells in pleural effusions express EMA and that this marker is useful, although not fully reliable as an indicator for malignancy. These data confirm the results reported by Croonen et al. (1988) who found EMA-positivity for pleural effusion cells of six malignant mesothelioma patients. Moreover, the detected EMA expression in malignant mesothelioma cells in pleural effusions is in line with the EMA expression in histologic specimen of malignant mesothelioma. The absence of EMA-expression reported by Battifora et al. (1985) could be due to usage of different antibodies against EMA. Experiments with different EMA- or Human Milk Fat Globule-specific antibodies

revealed that at least nine different epitopes can be distinguished (Hilkens et al., 1984).

Immunocytochemistry did not allow distinction between EMA-positive carcinoma or mesothelioma cells. However, malignant epithelial and malignant mesothelial cells can be discriminated on the basis of their ultrastructural features. In order to distinguish reactive from malignant mesothelial cells on the one hand and mesothelial from epithelial cells on the other hand immunoelectron microscopy with monoclonal antibodies against EMA was applied. The EMA antigen was found to be predominantly expressed on the outer surface of the villi of the malignant mesothelioma cells, while the villi of normal mesothelial cells were negative. The immunoelectron microscopical staining for EMA on pleural effusions of patients suspected of a malignant mesothelioma would be a useful addition to the standard diagnostic procedures. However, the technique is rather elaborate and therefore not applicable for routine pathology.

Cytogenetic aberrations in leukemia were found to have diagnostic and prognostic value (Yunis, 1983). In solid tumors also characteristic abnormalities with the possibility of diagnostic application have been detected (Teyssier, 1989). Moreover, these specific chromosomal aberrations have been found useful indicators for oncogenes involved in the various types of tumors. Cytogenetics of malignant mesothelioma were studied by several groups but mostly on solid tumor material and many patients had received therapy before analysis.

We investigated the cytogenetics of a groups of 40 malignant mesothelioma patients which were untreated at the time of analysis. Since diagnosis of malignant mesothelioma is difficult, diagnosis has been established by at least two of the following techniques: cytology, electron microscopy and histology. About 90% of the investigated specimen were pleural effusions. Our experience is that the direct method for cytogenetics of malignant mesothelioma is preferred above short term culture as overgrowth by normal cell types frequently was observed in these cultures. In 30 cases (27 pleural effusions) clonal abnormalities were found, once no metaphases were detected and in the other 9 cases only normal karyotypes were found. The detected aberrations were heterogeneous and of structural and numerical nature. However, no aberration was detected which was present in all cases investigated. In 4 cases normal karyotypes were found while cytological diagnosis of malignant mesothelioma was evident. The other 5 cases with normal metaphases were consistent with cytological findings or due to lack of material for the direct method.

Two patterns of clonal abnormalities were observed. In 24 of the 30 cases a hypodiploid or hypotetraploid karyotype was found with most frequently non-random loss of the chromosomes 4, 22 and partial deletion of 3p and 9p. Loss of suppressor genes, which are located on these chromosomes, could play a role in the development of a malignant mesothelioma. Further investigations should be performed in this

direction. The hypodiploid karyotype evolved after duplication or in one case cell fusion to hypotetraploidy. A similar pattern of evolution has been found in colon carcinoma (Muleris et al., 1987). The second pattern of abnormalities was a hyperdiploid karyotype with deletion of 3p and non-random gain of chromosomes 7, 5 and 20. This pattern was found in 6 of the 30 abnormal cases.

Cytogenetic analysis of malignant mesothelioma reported by others also showed a heterogeneous pattern of abnormalities and absence of a consistent marker. However, the pattern of abnormalities observed by several groups differ considerably. This can be due to other methods used for the analysis of the data, treatment of patients before analysis, passage in culture before analysis, exposure to different kinds of asbestos and genetic differences. The enormous amount of cytogenetic abnormalities found in malignant mesothelioma could possibly be the direct consequence of the asbestos exposure. Several investigators found *in vitro* chromosomal aberrations after asbestos exposure (Sincock et al., 1975). Particularly mesothelial cells were found to be sensitive to asbestos induced chromosomal aberrations (Lechner et al., 1985).

Diagnostic usefulness of the observed abnormalities was evaluated by cytogenetic analysis of effusions of non-malignant-, metastatic carcinoma-, and lung adenocarcinoma origin. Distinction between effusions of non-malignancy, metastatic carcinoma and malignant mesothelioma on the basis of patterns of cytogenetic aberrations was possible. However, no clear marker was found for the differentiation between lung adenocarcinoma and malignant mesothelioma. Furthermore, so far no correlation was observed between the cytogenetic pattern observed in malignant mesotheliomas and clinical data, like survival and histological type of tumor. But study of a larger group of patients might reveal such correlations.

Since human malignant mesothelioma cell lines were found to have an elevated c-*sis* expression (section 7.1), we investigated whether this observation is significant for the diagnosis of malignant mesothelioma. The detection of c-*sis* expression has to be performed on the mRNA level as no specific antibodies against the protein are available yet. Total RNA isolated from pleural effusion cells and tumor material of eight malignant mesothelioma patients was analysed by Northern blot for c-*sis* expression (section 7.2). Bands of similar size, specific for c-*sis*, were found in the tumor material of eight malignant mesotheliomas and two malignant mesothelioma cell lines isolated from two of these patients. No correlation between the level of c-*sis* expression and the morphologically detected percentage of tumor cells and/or macrophages was observed. This approach was hampered by the variable and frequently low incidence of tumor cells and the presence of other cells (e.g. macrophages) which can also express the c-*sis* oncogene (Martinet et al., 1986). The mRNA *in situ* hybridization technique offers the possibility to detect mRNA molecules at the single cell level. For

the discrimination between different c-*sis* positive cell types a double staining technique, which combines membrane antigen labeling with the detection of RNA transcripts is essential. This technique was recently developed (Bakkus et al., 1989a; b). Investigations in progress will reveal whether this technique is applicable for diagnostic purposes and whether the c-*sis* expression is a useful marker for the diagnosis of malignant mesothelioma. For this evaluation an mRNA in situ hybridization for c-*sis* in combination with a membrane antigen labeling specific for macrophages should be performed on pleural effusion cells of malignant mesothelioma patients, adenocarcinoma patients and patients without a malignancy. Nevertheless efforts should be directed towards the generation of antibodies that specifically recognize the different isoforms of PDGF. These antibodies will be preferred above the rather complicated RNA *in situ* hybridization technique and could be useful for the diagnosis of malignant mesothelioma. Furthermore they will allow the study of the role of different isoforms of PDGF *in vivo*.

8.2 Establishment and characterization of human malignant mesothelioma cell lines

The production of *in vitro* growing human malignant mesothelioma cell lines is essential for the study of malignant mesothelioma as viable tumor tissues are difficult to obtain and the incidence of tumor cells in pleural effusions is often very low (e.g. 5%). Furthermore, is the continuous availability and the constant quality of malignant mesothelioma cell lines an advantage for cell biological investigation.

In chapter 6 the production of a panel of human malignant mesothelioma cell lines from pleural effusions, biopsies and tumors obtained at autopsies is described. These *in vitro* growing malignant mesothelioma cell lines did not show signs of senescence, which is in contrast to cultured normal mesothelial cells that were found to have a limited lifespan. The processing of patient material and the culture conditions were described in section 6.1. The malignant mesothelioma cell lines were found to have a variable doubling time that was not influenced by freezing and thawing. Some cell lines have a doubling time of approximately 24 hours while others 48 hours. Because the growth of normal mesothelial cells was promoted by the addition of EGF and HC (Connell et al., 1983) the effect of the addition of these growth factors on the isolation of malignant mesothelioma cell lines was investigated. Since these growth factors were employed a number of malignant mesothelioma cell lines was isolated on medium with EGF and HC or EGF only while in most cases no growth was observed when the same cell samples were seeded in medium without these supplementations.

Although application of EGF and HC resulted in an elevated success-rate, their effect on individual samples was unpredictable. The growth of several malignant mesothelioma cell lines was stimulated by the addition of EGF to the culture medium

whereas EGF in combination with HC inhibited the growth of these cell lines. Another group of malignant mesothelioma cell lines was isolated on culture medium with EGF and HC while no growth was observed on medium with EGF only. In a single case growth occurred in all three culture media.

Only a few malignant mesothelioma cell lines have been described by others and no detailed data about the isolation procedure were given (Ohnuma et al., 1979; Behbehani et al., 1982; Tibbets et al., 1984; Shangfang et al., 1985; Anderson et al., 1986; Hsu et al., 1988). RPMI 1640 with different concentrations of serum was mostly used as culture medium, while in our study F10 medium was used for the isolation of malignant mesothelioma cell lines. Furthermore, other authors did not add EGF and HC or EGF only to their culture media. This could be the reason for the small number of malignant mesothelioma cell lines described in the literature.

A serious problem with the isolation of malignant mesothelioma cell lines is the determination whether the observed proliferation concerns malignant or benign mesothelial cells. Cytogenetic abnormality is the best indication for malignant cell growth. When cytogenetically abnormal cells are detected in the primary culture, immediate subcloning is necessary to obtain a 100% cytogenetically abnormal malignant mesothelioma cell line. The malignant mesothelioma character of isolated cell lines could also be determined by cytomorphological and ultrastructural studies. Moreover, the karyotype of the cell line has to be consistent with the aberrations detected in the original patient material.

Coexpression of cytokeratins and vimentin is a characteristic feature of mesothelial cells. Cytokeratins and vimentin were expressed in the malignant mesothelioma cell lines at a constant level independent of the growth rate of the cells. This is in contrast to normal mesothelial cells which were found to have reduced keratin and elevated vimentin levels during rapid growth. Reduction of the growth rate did increase the keratin content and decrease the vimentin levels (Connell et al., 1983). In section 6.2 the study of the individual cytokeratin polypeptides of three malignant mesothelioma cell lines is described. The three investigated malignant mesothelioma cell lines were found to be positive for the cytokeratins 7, 8, 18 and 19 by immunofluorescence and immunoblotting with chain specific monoclonal antibodies. This is in agreement with the results of Blobel (1985) who investigated histological specimen of malignant mesothelioma patients for their cytokeratin content. He found next to the cytokeratins 7, 8, 18 and 19 cytokeratin 5 in several epithelial and biphasic malignant mesotheliomas while this keratin was not expressed by normal mesothelial cells or lung adenocarcinomas. In the three investigated malignant mesothelioma cell lines, however, cytokeratin 5 was not detected in two dimensional gelelectrophoresis and immunoblotting.

The establishment and comparison of the properties of human malignant mesothelioma cell lines from different populations exposed to different types of asbestos fibers will be interesting. Especially the correlation between the cytogenetic characteristics of the tumor cells and the type of asbestos fibers to which the patient was exposed could possibly give more insight into the role of asbestos in the generation of malignant mesothelioma.

8.3 Expression of PDGF chains and PDGF receptors in human malignant mesothelioma cell lines

The panel of human malignant and normal mesothelial cell lines that was established has been used for the study of the expression of several genes involved in growth regulation. Section 7.1 describes the study of the messenger RNA expression of the PDGF A- and B-chain genes in ten malignant mesothelioma cell lines. These cell lines were found to have a strong expression of the PDGF B-chain or c-*sis* gene, which is barely detectable in normal mesothelial cells. The expression of the PDGF A-chain gene in the malignant mesothelioma cell lines was slightly elevated compared to normal mesothelial cells. These results are summarized in section 7.3, table 1. Our data confirm previous observations of Gerwin et al. (1987) concerning 7 mesothelioma cell lines and 4 normal mesothelial cell lines. All our investigated malignant mesothelioma cell lines, the ten described in section 7.1 and five which were recently isolated, were found to have an elevated but variable c-*sis* expression. In at least two of the seven investigated malignant mesothelioma cell lines of Gerwin et al. no c-*sis* expression was detected. It should be questioned whether these are indeed malignant mesothelioma cell lines as the origin and characteristics of these cell lines is unclear.

It seems that c-*sis* expression can be regarded as a consistent feature of malignant mesothelioma cell lines. This is in contrast with human osteosarcoma and melanoma cell lines, where c-*sis* expression was not detected in all cell lines investigated (Eva et al., 1982; Betsholtz et al., 1986; Westermark et al., 1986). For human malignant melanoma it was described that a cell line from the primary tumor had a much higher c-*sis* expression than cell lines from the metastases (Westermark et al., 1986). In our panel of malignant mesothelioma cell lines from primary and metastatic origin of the same patient no significant difference was detected in the level of c-*sis* expression (section 7.1).

In section 7.3 expression of PDGF A-type and PDGF B-type receptors on normal and malignant mesothelioma cell lines was studied. Expression of the PDGF A-type receptor was investigated by Northern blot analysis with a probe specific for the PDGF A-type receptor and binding experiments with ^{125}I-PDGF-AA. Normal mesothelial cells

were found to express the 7kb PDGF A-type receptor mRNA, while malignant mesothelioma cell lines hardly expressed this messenger. The binding of ^{125}I-PDGF-AA to the malignant mesothelioma cell lines varied from low to undetectable. Normal mesothelial cells could not be tested with this technique as these cells did not resist the extensive washing procedures. Since PDGF-AA can only bind to the PDGF A-type receptor these experiments indicate that PDGF A-type receptors are present at very low density on the eight investigated malignant mesothelioma cell lines. The presence of PDGF B-type receptors on normal and malignant mesothelial cell was demonstrated by Northern blot analysis, immunoprecipitation and binding experiments with radiolabeled PDGF-BB. We found in the malignant mesothelioma cell lines a variable level of expression of PDGF B-type receptor mRNA and in the normal mesothelial cell lines a weak to undetectable expression of this receptor (section 7.3).

The presence of PDGF B-type receptor protein in normal and malignant mesothelial cell lines was also demonstrated by immunoprecipitation with an antiserum specific for the PDGF B-type receptor. Experiments with ^{125}I-PDGF-BB revealed a specific binding of PDGF-BB to malignant mesothelioma cells. The binding of PDGF-BB to malignant mesothelioma cell lines indicates that functional receptors for this growth factor occur on the membrane of malignant mesothelioma cells. The results of the expression of the PDGF A-type and B-type receptor in normal and malignant mesothelial cells are summarized in section 7.3, table 1.

8.4 Regulation of the expression of genes coding for PDGF chains and PDGF receptors

In order to study the mechanism responsible for the elevated c-*sis* and PDGF A-chain gene expression the malignant mesothelioma cell lines were investigated for cytogenetic aberrations in the chromosomes 7 and 22, where the PDGF A- and B-chain genes are located, respectively. The number of copies of chromosome 7 was normal or increased and the number of copies of chromosome 22 was decreased. However, no specific chromosomal or genomic rearrangements were detected in the chromosomes 7 and 22. Gerwin et al. (1987) also did not find consistent changes. However, it cannot be excluded that DNA rearrangements outside the by Southern blot analysis investigated region are present. In order to detect these rearrangements inverse field gelelectrophoresis has to be performed. However, from other c-*sis* expressing cell lines no data are available that indicate towards such a mechanism of activation of the c-*sis* oncogene. Therefore activation of the c-*sis* oncogene on the transcriptional level has to be considered.

Recently some support for this hypothesis came from investigations of the K562 cell line. During induced megakaryocytic differentiation the c-*sis* mRNA level increased

200-fold (Alitalo et al., 1987; Pech et al., 1989). This induction of c-*sis* mRNA expression was found to be dependent of protein synthesis. Furthermore, a positive regulatory sequence in the c-*sis* promotor was found to bind nuclear factors and to function as a trans-activator in induced K562 cells and in a bladder carcinoma cell line that expressed the c-*sis* oncogene. However, the identified regulatory region was found not to be involved in the expression of the c-*sis* oncogene in two other tumor cell lines. Moreover, experiments with uninduced K562 cells indicate that the c-*sis* promotor is constitutively active in these cells. These results suggest that regulation beyond the level of transcription should be considered as well.

As the PDGF A- and B-type receptors are expressed at different levels in the normal and malignant mesothelial cell lines the regulation of the expression of these receptors is an important subject for further studies. Recently it was suggested that changes in isoform composition of the produced PDGF and in the type of PDGF receptor expressed could be a mechanism of regulation in the PDGF response (Bowen-Pope et al., 1989). This hypothesis was supported by observations in 3T3 cells. When these cells were exposed to TGFβ a decrease of PDGF A-type receptor expression and an increase of PDGF B-type expression was found (Bowen-Pope et al., 1989). These results indicate that PDGF A- and B-type receptor expression *in vitro* could be modulated by growth factors. It is unlikely that TGFβ plays a similar role in PDGF receptor expression in malignant mesothelioma cell lines as both normal and malignant mesothelial cells were found to secrete approximately similar amounts of active TGFβ (Gerwin et al., 1987). However, it has to be studied whether e.g. EGF and HC that is added to the normal mesothelial cultures plays a role in PDGF receptor expression in normal and malignant mesothelial cell lines.

In several malignant mesothelioma cell lines an inverse relationship between c-*sis* and PDGF B-type receptor mRNA was observed (section 7.2). This is in contrast with results obtained from SSV transformed fibroblasts in which high levels PDGF B-type receptor were found to correlate with high levels of *sis* gene product, while non-transformed cells did not have a rapid turnover of the PDGF B-type receptor (Huang and Huang, 1988). However, comparison of PDGF B-type receptor and c-*sis* levels in normal and malignant cells is in agreement with this observation. Analysis of the effect of transfection of the PDGF B-type receptor gene and/or c-*sis* gene on the level of c-*sis* and PDGF B-type mRNA in normal and malignant mesothelial cells might reveal how the expression of these genes is related to each other.

8.5 Autocrine stimulation

Many data supporting the autocrine stimulation model were obtained in SSV transformed cells. It has been demonstrated that only cell types that have functional

PDGF receptors are susceptible for SSV transformation (Leal et al., 1985). In these cells next to expression of the PDGF B-chain the presence of the appropriate growth factor receptor is considered a prerequisite for an autocrine function of the produced PDGF. In the malignant mesothelioma cell lines next to PDGF B-chain and PDGF B-type receptor expression also the PDGF A-chain gene is expressed, while the PDGF A-type receptor is absent or present in very low amounts. Expression of the PDGF A-chain in combination with B-chain and B-type receptor expression was also found in freshly obtained human gliomas (Hermansson et al., 1988). However no data about PDGF A-type receptor expression were available and the PDGF A-chain gene was expressed at a much higher level than the B-chain and B-type receptor genes. Until now the role of PDGF A-chains in malignant mesotheliomas is unclear. It is possible that A- and B-chains are assembled as heterodimers that can function as autocrine or paracrine growth factors. However, the possibility that AB heterodimers can function as an autocrine growth factor in malignant mesothelioma cells, which have no or very low levels of A-type receptors, is unlikely according to the "PDGF receptor subunit model" (Chapter 2.5) and recent observations in human fibroblasts. Analysis of the differential effect of the three isoforms of PDGF on several functional parameters in these cells indicate that PDGF-AB binds to and dimerizes an A- and B-type receptor (A. Hammacher et al., submitted). Thus AB heterodimers produced by malignant mesothelioma cells probably have a paracrine effect (Fig. 1.1). Another possibility is that BB-homodimers are produced in combination with AA and/or AB-dimers (Figs. 1.2, 1.3 and 1.4). The produced BB can function as an autocrine growth factor or just as the AA and/or AB dimers, have a paracrine effect. The paracrine effect of the different isoforms of PDGF that could be produced by the malignant mesothelioma cells may be on surrounding connective tissues cells and normal mesothelial cells. To investigate the latter possibility effects of exogenous addition of different isoforms of PDGF on cultured normal mesothelial cells should be studied. An observation that indicates towards a role of B-chain production in the transformation of mesothelial cells is made in SV40 large T-antigen transfected normal mesothelial cells. In these cells expression of the PDGF B-chain gene was observed, while there was no change in the level of PDGF A-chain gene expression compared to normal mesothelial cells (Gerwin et al., 1987). Though these cells were not immortalized they were found to have a prolonged lifespan compared to normal mesothelial cells. Moreover, a function of PDGF-BB as an autocrine growth factor in malignant mesothelioma is supported by the striking difference in PDGF B-chain expression between normal mesothelial cells and malignant mesothelioma cells and the expression of the appropriate receptor for this growth factor.

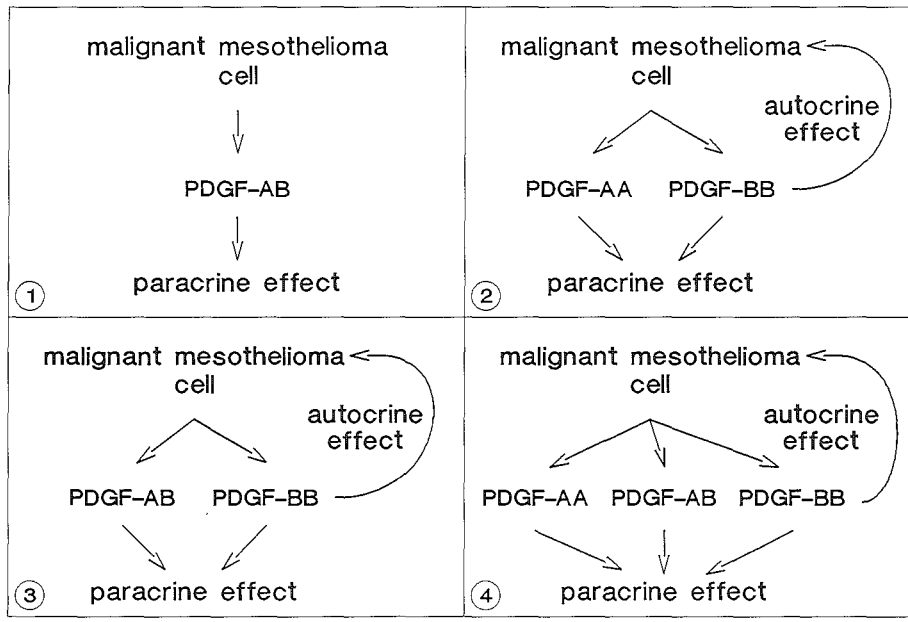

Figure 1. *Possible effects for the different PDGF molecules that could be produced by malignant mesothelioma cell lines.*

It is presently unclear whether interaction between the c-*sis* gene product and the PDGF B-type receptor takes place via an extracellular loop or is an intracellular interaction. In the malignant mesothelioma cell lines immunoelectron microscopical investigations with antibodies against the PDGF B-type receptor revealed that although plasma membrane associated PDGF B-type receptors occur, also receptors are present in presumptive vesicles and lysosomes in the cytoplasm. These results are consistent with observations in *sis*-transformed NRK cells and fibroblasts (Keating et al., 1988; Huang and Huang, 1988). The majority of the newly produced PDGF receptors in these cells do not reach the cell surface and are rapidly degraded. It was suggested that this was due to interaction of the *sis* gene product with the receptor in the endoplasmic reticulum and/or Golgi apparatus in contrast with the hypothesis that the c-*sis* product is secreted and binds to receptors at the cell surface. The first hypothesis is supported by the observation by Robbins et al. (1985) that the major part of the v-*sis* gene product remained cell associated and that only a small amount was secreted. Another observation that supports an intracellular interaction is that anti-PDGF-antibodies could only partially inhibit the proliferation of some SSV-transformed cells and were unable to reverse the transformed phenotype of these cells completely

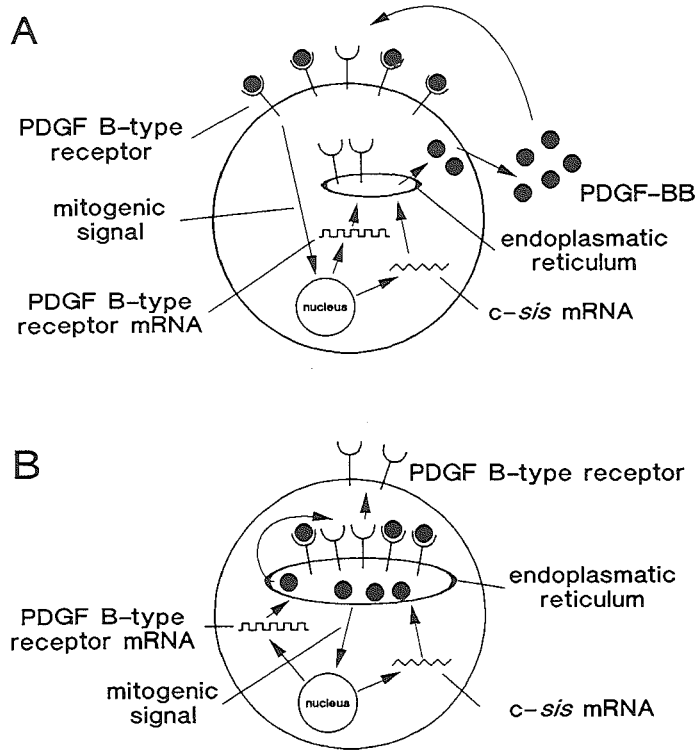

Figure 2. *Autocrine stimulation by an extracellular and intracellular pathway. A. Extracellular autocrine pathway for the c-sis gene product. B. Intracellular interaction between the gene product of c-sis and its receptor.*

(Huang et al., 1984; Johnsson et al., 1985). Two findings in malignant mesothelioma cell lines support an intracellular interaction between c-*sis* and its receptor. First, the three malignant mesothelioma cell lines investigated so far were also found to secrete minor amounts of PDGF (M.A. Versnel et al., unpublished results). Secondly, immunoelectron microscopic investigations revealed that PDGF B-type receptors are present in presumptive vesicles in the cytoplasm of malignant mesothelioma cells (section 7.3). The expression of PDGF A- and PDGF B-chain genes and the presence of PDGF B-type receptors in human malignant mesothelioma cell lines indicates that

PDGF BB indeed can function as an autocrine growth factor. Furthermore, intracellular interaction between the produced PDGF and its receptor should be considered (Fig. 2).

The role of PDGF chains and PDGF receptors in the malignant transformation of mesothelial cells can be further elucidated by several approaches. The effect of exogenous addition of different isoforms of PDGF on functional parameters that are known to be involved in PDGF response in other cell types has to be studied. Another approach is to transfect mesothelial cells with constructs of PDGF chain- and PDGF-receptor genes and to analyse their effect on cell growth and other parameters.

Investigations of a possible autocrine loop of PDGF-BB in malignant mesothelioma cell lines should be directed towards specific interference somewhere in the autocrine pathway. Suramin is an agent that interferes with the binding of PDGF to its receptor. The proliferation of five malignant mesothelioma cell lines was found to be inhibited by suramin addition to the culture medium (M.A. Versnel, unpublished results). Since the effect of suramin is not specific for the interaction of PDGF with its receptor but for other growth factor-receptor interactions, as well more specific methods for interference should be developed. A possibility is interference at the mRNA level which can be approached by transfection with an antisense *sis* or PDGF B-type receptor construct under the control of a strong promotor. Another possibility is to block the interaction between the receptor and growth factor at the protein level by antibodies against the binding domain of the PDGF B-type receptor or antibodies that bind to PDGF-BB in such a way that interaction with the receptor is no longer possible.

If the data concerning the PDGF chain and receptor expression in malignant mesothelioma cell lines correspond to the *in vivo* situation and PDGF is an autocrine growth factor that interacts with membrane bound PDGF receptors on these cells intrapleural application of a PDGF antagonist could have therapeutic consequences for malignant mesotheliomas.

8.6 References

Alitalo, R., Andersson, L.C., Betsholtz, C., Nilsson, K., Westermark, B., Heldin C.-H. and Alitalo, K. Induction of platelet-derived growth factor gene expression during megakaryoblastic and monocytic differentiation of human leukemia cell lines. EMBO J. 6, 1213-1218, 1987.

Anderson, T.M., Shin, W.-Y., Benedict, W.F., Holmes, E.C. and Saxton, R.E. Establishment of a human mesothelioma cell line. Proc. Am. Assoc. Cancer Res. 27, 31, 1986.

Battifora, H. Recent progress in the immunohistochemistry of solid tumors. Semin. Diagn. Pathol. 1, 251-271, 1984.

Bakkus, M.H.C., Versnel, M.A., Adriaansen, H.J. and Van den Akker, Th.W. Non-isotopic RNA *in situ* hybridization: The detection of low copy number mRNA molecules in bone marrow cells of multiple myeloma patients. In: Monoclonal gammapathies. II. Clinical significance and basic mechanisms. Radl, J. and Van Camp, B. (Eds.) 1989a, pp. 113-116.

Bakkus, M.H.C., Brakel-van Peer, C.M.J., Adriaansen, H.J., Wierenga-Wolf, A.F., van den Akker, Th.W., Dicke-Evinger, M.J. and Benner, R. Detection of oncogene expression by

fluorescent *in situ* hybridization in combination with immunofluorescent staining of cell surface markers. Oncogene, in press, 1989b.
Battifora, H. and Kopinski, M.T. Distinction of mesothelioma from adenocarcinoma: An immunohistochemical approach. Cancer 55, 1679-1685, 1985.
Behbehani, A.M., Hunter, W.J., Chapman, A.L. and Lin, F. Studies of a human mesothelioma. Hum. Pathol. 13, 862-866, 1982.
Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L. Knott, T.J. and Scott, J. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. Nature 320, 695-699, 1986.
Blobel, G.A., Moll, R., Franke, W.W., Kayser, K.W. and Gould, V.E. The intermediate filament cytoskeleton of malignant mesotheliomas and its diagnostic significance. Am. J. Pathol. 121, 235-247, 1985.
Bowen-Pope, D.F., Hart, C. and Seifert, R.A. Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which bind to different classes of PDGF receptor. J. Biol. Chem. 264, 2502-2508, 1989.
Connell, N.D. and Rheinwald, J.G. Regulation of the cytoskeleton in mesothelial cells: reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell 34, 245-253, 1983.
Croonen, A.M., Van der Valk, P., Herman, C.J. and Lindeman, J. Cytology, immunopathology and flow cytometry in the diagnosis of pleural and peritoneal effusions. Lab. Invest. 58, 725-732, 1988.
Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C. and Aaronson, S.A. Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells. Nature 295, 116-119, 1982.
Gerwin, B.I., Lechner, J.F., Reddel, R.R., Roberts, A.B., Robbins, K.C., Gabrielson, E.W. and Harris, C.C. Comparison of production of transforming growth factor-β and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47, 6180-6184, 1987.
Gosh, A.K., Mason, D.Y. and Spriggs, A.I. Immunocytochemical staining with monoclonal antibodies in cytologically "negative" serous effusions from patients with malignant disease. J. Clin. Pathol. 36, 1150-1152, 1983.
Hammacher, A., Mellström, K., Heldin, C.-H. and Westermark, B. Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer. Submitted.
Hermansson, M., Nister, M., Betsholtz, C., Heldin, C.-H., Westermark, B. and Funa, K. Endothelial cell hyperplasia in human glioblastoma: coexpression of mRNA for platelet-derived growth factor (PDGF) B chain and PDGF receptor suggests autocrine growth stimulation. Proc. Natl. Acad. Sci. USA 85, 7748-7752, 1988.
Hilkens, J., Buys, F., Hilgers, J., Hageman, P., Calafat, J., Sonnenberg, A. and Van der Valk, M. Monoclonal antibodies against human milk-fat globule membranes detecting differentiation antigens of the mammary gland and its tumors. Int. J. Cancer 34, 375-379, 1984.
Hsu, S.-M., Hsu, P.-L., Zhao, X., Kao-Shan, C.S. and Whang-Peng, J. Establishment of human mesothelioma cell lines (MS-1, -2) and production of a monoclonal antibody (Anti-MS) with diagnostic and therapeutic potential. Cancer Res. 48, 5228-5236, 1988.
Huang, J.S., Huang, S.S. and Deuel, Th.F. Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors. Cell 39, 79-87, 1984.
Huang, S.S. and Huang, J.S. Rapid turnover of the platelet-derived growth factor receptor in *sis*-transformed cells and reversal by suramin; implications for the mechanism of autocrine transformation. J. Biol. Chem. 263, 12608-12618, 1988.
Johnsson, A., Betsholtz, C., Heldin, C.-H. and Westermark, B. Antibodies against platelet-derived growth factor inhibit acute transforming by simian sarcoma virus. Nature 317, 438-440, 1985.
Keating, M.T. and Williams, L.T. Processing of the platelet-derived growth factor receptor. J. Biol. Chem. 262, 7932-7937, 1987.
Leal, F., Williams, L.T., Robbins, K.C. and Aaronson, S.A. Evidence that the v-*sis* gene product transforms by interaction with the receptor for platelet-derived growth factor. Science 230, 327-330, 1985.
Lechner, J.F., Tokiwa, T., LaVeck, M., Benedict, W.F., Banks-Schlegel, S., Yaeger, H., Banerjee, A. and Harris, C.C. Asbestos-associated chromosomal changes in human mesothelial cells. Proc. Natl. Acad. Sci. USA 82, 3884-3888, 1985.

Marshall, R.J., Herbert, A., Braye, S.G. and Jones, D.B. Use of antibodies to carcino-embryonic antigen and human milk fat globule to distinguish carcinoma, mesothelioma, and reactive mesothelium. J. Clin. Pathol. 37, 1215-1221, 1984.

Martinet, Y., Bitterman, P.B., Mornex, J.-F., Grotendorst, G.R., Martin, G.R. and Crystal, R.G. Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity. Nature 319, 158-160, 1986.

Muleris, M., Salmon, R.J., Dutrillaux, A.M., Viehl, P., Zafranib, B. and Girodet, J. Characteristic chromosomal imbalances in 18 near-diploid colorectal tumors. Cancer Genet. Cytogenet. 29, 289-301, 1987.

Ohnuma, T., Wolman, S.R., Daum, S.M. and Holland, J.F. Establishment and characterization of a human mesothelioma cell line. Proc. Am. Assoc. Cancer Res. 20, 162, 1979.

Pech, M., Rao, C.D., Robbins, K.C. and Aaronson, S.A. Functional identification of regulatory elements within the promoter region of platelet-derived growth factor 2. Molec. Cell. Biol. 9, 396-405, 1989.

Pinkus, G.S. and Kurtin, P.J. Epithelial membrane antigen: A diagnostic discriminant in surgical pathology: Immunohistochemical profile in epithelial, mesenchymal and hematopoietic neoplasms using parafin sections and monoclonal antibodies. Hum. Pathol. 16, 929-940, 1985.

Robbins, K.C., Leal, F., Pierce, J.H. and Aaronson, S.A. The v-*sis*/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. EMBO J. 4, 1783-1792, 1985.

Shanfang, W., Enzhong, W., Jianzhong, S., Huixuan, X., Zhenqiong, L., Cifu, S., Changwen, X., Ruiming, C., Xiuzhen, Y., Dehou, Z. and Zhenhong, Y. Establishment and characterization of human malignant pleural mesothelioma cell line SMC-1. Sci. Sin. 28, 281-288, 1985.

Sincock, A. and Seabright, M. Induction of chromosome changes in Chinese hamster cells by exposure to asbestos. Nature 257, 56-58, 1975.

Sloane, J.P., Hughes, F. and Ormerod, M.G. An assessment of the value of epithelial membrane antigen and other epithelial markers in solving diagnostic problems in tumour histopathology. Histochem. J. 15, 645-654, 1983.

Teyssier, J.R. The chromosomal analysis of human solid tumors. A triple challenge. Cancer Genet. Cytogenet. 37, 103-125, 1989.

Tibbets, L.M., Chu, M.Y., Spremulli, E.N., Leduc, E.H. and Calabresi, P. Loss and restoration of tumorgenicity in a human mesothelioma cell line. Proc. Am. Assoc. Cancer Res. 25, 45, 1984.

To, A., Coleman, D.V., Dearnaley, D.P., Ormerod, M.G., Stelle, K. and Neville, A.M. Use of antisera to epithelial membrane antigen for the cytodiagnosis of malignancy in serous effusions. J. Clin. Pathol. 34, 1326-1332, 1981.

To, A., Dearnaley, D.P., Ormerod, M.G., Canti, G. and Coleman, D.V. Epithelial membrane antigen: its use in the diagnosis of malignancy in serous effusions. Am. J. Clin. Pathol. 78, 214-219, 1982.

Westermark, B., Johnsson, A., Paulsson, Y., Betsholtz, C., Heldin, C.-H., Herlyn, M., Rodeck, U. and Koprowski, H. Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor. Proc. Natl. Acad. Sci. 83, 7197-7200, 1986.

Yunis, J.J. The chromosomal basis of human neoplasia. Science 221, 227-236, 1983.

SUMMARY

Human malignant mesothelioma is a tumor of the coelomic cavities, that originates from mesodermally derived tissues. Malignant mesotheliomas most frequently are found in the pleura. The occurrence of malignant mesothelioma is strongly associated with asbestos exposure. Although in recent years asbestos usage has been greatly deminished the incidence of malignant mesothelioma is still increasing. This is due to the long latent period (15-45 years) between asbestos exposure and the development of the tumor.

In this thesis clinical, diagnostic and cell biological aspects of human malignant mesothelioma were studied. The purpose of these studies was to improve the diagnosis of human malignant mesothelioma on the one hand and to investigate the growth regulation and transformation of normal and malignant mesothelial cells on the other hand.

For this investigation material was collected of malignant mesothelioma patients form the areas of Rotterdam and Vlissingen in the Netherlands. A retrospective study of the medical records of 124 malignant mesothelioma patients is described in chapter 4. A history of asbestos exposure, with a median duration of 33.6 years was found in 91% of the patients. The median latent period was 41.2 years and the median survival after diagnosis 11 months. Radiologic investigations showed most frequently the presence of a pleural effusion.

Diagnosis of malignant mesothelioma on pleural effusion cells is difficult because malignant mesothelioma cells lack specific characteristics that can be used for the differentiation from adenocarcinoma cells and reactive mesothelial cells. Therefore we investigated the usefulness of immunoelectron microscopy and cytogenetics for diagnostic application on pleural effusion cells (chapter 5).

Expression of epithelial membrane antigen (EMA) on malignant mesothelioma cells of 25 patients was studied by immunocytochemistry. In 23 cases EMA positive cells were detected and in two such cells were not found. Immunoelectron microscopy revealed that EMA was localized predominantly on the villi of the malignant mesothelioma cells. The combination of ultrastructural investigations with immunogold staining for EMA enabled us to discriminate between malignant mesothelioma cells and malignant epithelial and reactive mesothelial cells.

Cytogenetic analysis of 40 malignant mesotheliomas revealed complex karyotypic abnormalities in 30 cases, in 9 cases the karyotype found was normal and in a single case the metaphases could not be analysed. The chromosomal abnormalities were heterogeneous and of structural and numerical nature. Although two patterns of non-random changes were observed no consistent specific abnormality was found. In 24 of the 30 cases a hypodiploid or hypotetraploid karyotype with loss of the chromosomes 4, 22, 9p and 3p was found. In 6 cases a hyperdiploid karyotype with deletion of 3p and gain of the chromosomes 7, 5, and 20 was found. The diagnostic significance of the abnormalities observed in malignant mesothelioma was investigated by comparison with the cytogenetics from lung adenocarcinoma, metastatic carcinoma and non-malignant pleural effusions. The latter two types of pleural effusion could be distinguished by cytogenetics from malignant mesothelioma. However, differentiation between lung adenocarcinoma and malignant mesothelioma by cytogenetics was not possible.

The production of *in vitro* growing malignant mesothelioma cell lines is a prerequisite for cell biological study of malignant mesothelioma as viable tumor tissues are difficult to obtain and the incidence of tumor cells in pleural effusions is often very low. The method used for the establishment of 17 malignant mesothelioma cell lines from 61 samples of patient material is described in chapter 6.1. To determine during the establishment whether the observed proliferation is of malignant mesothelioma cells or contaminating normal mesothelial cells cytogenetic abnormality was found to be the best indicator for malignant cell growth. The addition of epidermal growth factor (EGF) and hydrocortisone (HC) to the culture medium increased the chance of a successful isolation. However, the effect of addition of these growth factors on individual samples was impredictable.

The characteristics of three malignant mesothelioma cell lines (Mero-14, Mero-25 and Mero-41) are described in chapter 6.2. Light and electron microscopical investigations revealed that the morphology of the cell lines is consistent with the mesothelial nature of the cells. All three cell lines had a hyperdiploid karyotype similar to that found in the original patient material. Addition of EGF to the culture medium caused an increase of the proliferation of the cell lines Mero-14 and Mero-25. A decreased proliferation was found after addition of HC or EGF plus HC. In all three cell lines expression of the cytokeratins 7, 8, 18 and 19 was detected by immunofluorescence and immunoblotting with chain specific monoclonal antibodies. These characteristics show that the cell lines are derived from malignant mesotheliomas and have retained their original character.

The expression of PDGF chain and PDGF receptor genes in malignant mesothelioma cell lines is described in chapter 7. Ten human malignant mesothelio-

ma cell lines appeared to have an elevated expression of the PDGF B-chain or c-*sis* gene compared to the barely detectable level of mRNA expression in normal mesothelial cells. Expression of the PDGF A-chain gene in malignant mesothelioma cells was found to be slightly elevated compared to normal mesothelial cells. Subsequently, we investigated whether PDGF B-chain gene expression is also present in freshly obtained tumor material. Total RNA was isolated from tumor cells of eight malignant mesothelioma patients and analysed by Northern blotting for PDGF B-chain gene expression (chapter 7.2). In these samples a variable level of expression of the PDGF B-chain was observed. This expression did not correlate with the morphologically detected percentage tumor cells.

For a possible function of the by malignant mesothelioma cells produced PDGF as an autocrine growth factor, expression of PDGF receptors is thought a prerequisite. Therefore we investigated the expression of PDGF A- and B-type receptors in malignant and normal mesothelial cells (chapter 7.3). Malignant mesothelioma cell lines expressed PDGF B-type receptor mRNA and protein, while the PDGF A-type receptor mRNA was hardly detectable. In contrast, cultured normal mesothelial cells express PDGF A-type receptor mRNA and had a low to undetectable levels of PDGF B-type receptor mRNA and protein expression. The expression of PDGF A- and B-chain genes and the presence of PDGF receptors on malignant mesothelioma cell lines suggest that the produced PDGF could function as an autocrine growth factor and play a role in the malignant transformation of mesothelial cells.

The studies described in this thesis have enlarged our knowledge about the characteristics of malignant mesothelioma cells. The isolated panel of malignant mesothelioma cell lines will be a useful tool for the further development of diagnostic techniques. Furthermore, cultured normal and malignant mesothelial cells are a useful model for the study of the regulation of the expression of PDGF chain- and PDGF receptor genes.

SAMENVATTING

Het humane maligne mesothelioom is een tumor van de coeloomholte, die ontstaat uit weefsels van mesodermale oorsprong. Maligne mesotheliomen worden het meest frequent in de pleura gevonden. Het ontstaan van een maligne mesothelioom wordt sterk geassocieerd met blootstelling aan asbest. Hoewel het gebruik van asbest de laatste jaren sterk verminderd is, neemt de incidentie van het maligne mesothelioom nog steeds toe. Dit wordt veroorzaakt door de lange latentie periode (15-45 jaar) die ligt tussen de blootstelling aan asbest en het ontstaan van de tumor.

In dit proefschrift worden de klinische, diagnostische en celbiologische aspekten van het maligne mesothelioom bestudeerd. Het doel van deze studie was verbetering van de diagnostiek van het maligne mesothelioom enerzijds en onderzoek van de groeiregulatie en transformatie van normale en maligne mesotheelcellen anderzijds.

Voor deze studie werd materiaal van maligne mesothelioom patiënten uit de regio's Rotterdam en Vlissingen verzameld. Een retrospectief onderzoek van de medische gegevens van 124 maligne mesothelioom patiënten wordt in hoofdstuk 4 beschreven. Asbestblootstelling met een gemiddelde duur van 33,6 jaar werd in het verleden van 91% van de patiënten gevonden. De gemiddelde latentieperiode was 41,2 jaar en de gemiddelde overleving na diagnose 11 maanden. Radiologisch onderzoek toonde meestal de aanwezigheid van pleuravocht aan.

De diagnostiek van het maligne mesothelioom op pleuravochtcellen is moeilijk omdat maligne mesothelioomcellen geen specifieke eigenschappen hebben die onderscheid tussen adenocarcinoomcellen en reaktieve mesothelioomcellen mogelijk maken. De bruikbaarheid van immunoelectronen microscopische en cytogenetische analyse voor diagnostische toepassing op pleuravochtcellen wordt besproken in hoofdstuk 5. Expressie van het epitheliaal membraan antigeen (EMA) op maligne mesothelioomcellen van 25 patiënten werd bestudeerd met immunocytochemie en immunoelectronen microscopie. In 23 gevallen werden EMA positieve cellen gedetecteerd en in twee werden dergelijke cellen niet gevonden. Immunoelectronen microscopie toonde aan dat EMA voornamelijk gelokaliseerd was op de villi van de maligne mesothelioomcellen. De combinatie van ultrastruktureel onderzoek met immunogoud kleuring van EMA stelde ons in staat maligne mesothelioomcellen te onderscheiden van maligne epitheelcellen en reaktieve mesotheelcellen.

Cytogenetisch onderzoek van 40 maligne mesotheliomen toonde complexe karyotypische afwijkingen aan in 30 gevallen; in 9 gevallen was het gevonden karyogram normaal en éénmaal konden de metafasen niet geanalyseerd worden. De chromosomale afwijkingen waren heterogeen en van strukturele en numerieke aard. Hoewel twee patronen van non-random veranderingen werden waargenomen, is geen consistente specifieke afwijking gevonden. In 24 van de 30 gevallen werd een hypodiploïd of hypotetraploïd karyotype gevonden, waarbij verlies van de chromosomen 4, 22, 9p en 3p optrad. In 6 gevallen werd een hyperdiploïd karyogram gevonden met deletie van 3p en een toename van het aantal chromosomen 7, 5 en 20. Het diagnostische belang van de in maligne mesotheliomen waargenomen afwijkingen werd onderzocht door cytogenetisch onderzoek van pleuravocht van longadenocarcinomen, metastatische carcinomen en niet-maligne aandoeningen. De laatste twee typen pleuravocht konden met behulp van cytogenetica van maligne mesotheliomen onderscheiden worden. Echter, onderscheid tussen long adenocarcinomen en maligne mesotheliomen met behulp van cytogenetica bleek niet mogelijk.

De produktie van *in vitro* groeiende maligne mesothelioom cellijnen is een voorwaarde voor celbiologisch onderzoek van het maligne mesothelioom aangezien vitaal tumormateriaal moeilijk te verkrijgen is en de incidentie van tumorcellen in pleuravocht vaak erg laag is. De methode die is gebruikt voor het produceren van 17 maligne mesothelioom cellijnen uit 61 monsters van patiëntenmateriaal wordt beschreven in hoofdstuk 5.1. Om gedurende de ontwikkeling van een maligne mesothelioomcellijn te bepalen of de waargenomen proliferatie van de maligne mesothelioomcellen is of van contaminerende normale mesotheelcellen was een afwijkend karyogram de beste indicator voor maligne celgroei. De toevoeging van epidermal growth factor (EGF) en hydrocortison (HC) aan het kweekmedium verhoogde de kans op een succesvolle isolatie. Echter, het effekt van de toevoeging van deze groeifaktoren op individuele monsters is onvoorspelbaar. De karakteristieken van drie maligne mesothelioomcellijnen (Mero-14, Mero-25 en Mero-41) worden beschreven in hoofdstuk 6.2. Licht- en electronenmicroscopisch onderzoek toonden aan dat de morfologie van deze cellijnen overeenkomt met de mesotheliale oorsprong van de cellen. Alle drie cellijnen hadden een hyperdiploïd karyogram dat hetzelfde was als in het oorspronkelijke patiëntenmateriaal. Toevoeging van EGF aan het kweekmedium veroorzaakte een toename van de proliferatie van de cellijnen Mero-14 en Mero-25. Een afname van de proliferatie werd gevonden na toevoeging van HC of EGF en HC. In alle drie de cellijnen werd expressie van de cytokeratines 7, 8, 18 en 19 met behulp van immunofluorescentie en immunoblotting met keten-specifieke antilichamen gevonden. Deze eigenschappen tonen aan dat de cellijnen afkomstig zijn van maligne mesotheliomen en hun oorspronkelijk karakter hebben behouden.

De expressie van genen die coderen voor de platelet-derived growth factor (PDGF) en PDGF receptoren in maligne mesothelioomcellijnen wordt beschreven in hoofdstuk 7. Tien humane maligne mesothelioomcellijnen bleken een verhoogde expressie van het PDGF B-keten gen of c-*sis* gen te hebben ten opzichte van het nauwelijks te detecteren niveau van mRNA expressie in normale mesotheelcellen. Expressie van het PDGF A-keten gen in maligne mesothelioomcellijnen was licht verhoogd ten opzichte van normale mesotheelcellen. Vervolgens onderzochten wij of de PDGF B-keten genexpressie ook in vers tumormateriaal aanwezig was. Totaal RNA werd geïsoleerd uit materiaal van acht maligne mesothelioompatiënten en met behulp van de Northern blot analyse voor PDGF B-keten gen expressie onderzocht (hoofdstuk 7.2). In deze monsters werd een variabel niveau van PDGF B-keten gen expressie gevonden dat niet correleerde met het morfologisch gedetecteerde aantal tumorcellen.

De door maligne mesothelioomcellen geproduceerde PDGF zou als een autocriene groeifaktor kunnen funktioneren als maligne mesothelioomcellen PDGF receptoren tot expressie brengen. Daarom hebben we de expressie van PDGF A-type en B-type receptoren in maligne en normale mesotheelcellen onderzocht (hoofdstuk 7.3). Maligne mesothelioomcellijnen brachten PDGF B-type receptor mRNA en eiwit tot expressie, terwijl PDGF A-type receptor mRNA nauwelijks te detecteren was. In tegenstelling hiermee brachten gekweekte normale mesotheelcellen PDGF A-type receptor mRNA tot expressie en hadden een lage tot niet detecteerbare PDGF B-type mRNA en eiwit expressie. De expressie van PDGF A- en B-keten genen en de aanwezigheid van PDGF receptoren geeft aan dat de geproduceerde PDGF kan funktioneren als een autocriene groeifaktor en zo een rol kan spelen in de maligne transformatie van mesotheelcellen.

Het onderzoek dat in dit proefschrift wordt beschreven heeft de kennis vergroot over de eigenschappen van maligne mesothelioomcellen. Het geïsoleerde panel van maligne mesothelioomcellijnen zal een bruikbaar instrument zijn voor de ontwikkeling van nieuwe diagnostische technieken. Bovendien zullen gekweekte normale en maligne mesotheelcellen een nuttig model zijn voor onderzoek naar de regulatie van de expressie van genen die coderen voor PDGF ketens en PDGF receptoren.

CURRICULUM VITAE

24 juni 1958	Geboren te Utrecht
juni 1976	Eindexamen atheneum-B, Thorbecke Scholengemeenschap, Utrecht
september 1978	Aanvang studie Biologie
september 1981	Kandidaatsexamen Biologie, B1 Rijksuniversiteit Utrecht
februari 1984	Doctoraalexamen Biologie, Rijksuniversiteit Utrecht
	Hoofdvakken: - Moleculaire Genetica Prof. Dr. G.A. van Arkel Rijksuniversiteit Utrecht Prof. Dr. D. Bootsma Erasmus Universiteit Rotterdam - Immunologie Prof. Dr. R. Benner Erasmus Universiteit Rotterdam
	Bijvak: Didaktiek van de Biologie, 1e graads lesbevoegdheid Rijksuniversiteit Utrecht
maart 1984	Aanvang promotieonderzoek op de afdeling Celbiologie, Immunologie en Genetica, Erasmus Universiteit Rotterdam bij Prof. Dr. R. Benner en Prof. Dr. A. Hagemeijer

LIST OF PUBLICATIONS

J.J.M. van Dongen, I.L.M. Tettero, A.H.M. Geurts van Kessel, M.A. Versnel, H. Hooijkaas and A. Hagemeijer. Confirmation of the assignment of the gene coding for the BA-2 antigen to chromosome 12. Hum Genet. 68: 351-352, 1984.

J.J.M. van Dongen, M.A. Versnel, A.H.M. Geurts van Kessel, E.M.E. Smit, H. Hooijkaas and A. Hagemeijer. Immunoglobulin chain expression in Burkitt Lymphoma derived cell lines. In: Leukemia Recent Advances in Biology and Treatment 1985, p. 481-489, Alan R. Liss Inc.

M.A. Versnel, J.J.M. van Dongen, A.H.M. Geurts van Kessel, A. de Klein, N.A. Bos and A. Hagemeijer. Expression of the human immunoglobulin heavy chain gene of the 14q+ chromosome in a t(8;14)-positive Burkitt lymphoma cell line demonstrated in somatic cell hybrids. Cancer Genet. Cytogenet. 19: 321-330, 1986.

B. Tank, G. van Dijk, J. van der Sluis, A.W.M. van der Kamp, D. Schonk, E. Stolz, M.A. Versnel and Th. van Joost. Detection of basal-cell determinant(s) in human psoriatic skin: a monoclonal antibody study. Arch. Derm. Res. 279: 68-70, 1986.

J.J.M. van Dongen, A.H.M. Geurts van Kessel, I.L.M. Wolvers-Tettero, M.A. Versnel, A. van Oudenaren, E. Schoenmaker and A. Hagemeijer. Construction of human-mouse T cell hybrids that express human T cell-associated surface antigens and alow the chromosomal localization of the antigens. J. Immunol. 137: 1047-1053, 1986.

Th.H. van der Kwast, M.A. Versnel, M. Delahaye, A. de Jong, P.E. Zondervan and H.C. Hoogsteden. Expression of epithelial membrane antigen on malignant mesothelioma cells. Acta Cytol. 32: 169-174, 1988.

Th.H. van der Kwast, V.D. Vuzevski, M.A. Versnel, M. Delahaye, A. de Jong and H.C. Hoogsteden. Ultrastructural localization of Epithelial Membrane Antigen and Carcinoembryonic Antigen in malignant mesothelial and epithelial cells. In: New Frontiers in Cytology. Goertter, K., Feichter, G.E. and Witte, S. (Eds.) 1988, p. 265-273, Springer-Verlag.

M.A. Versnel, A. Hagemeijer, M.J. Bouts, Th.H. van der Kwast and H.C. Hoogsteden. Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene 2, 601-605, 1988.

M.H.C. Bakkus, M.A. Versnel, H.J. Adriaansen and Th.W. van den Akker. Non-isotopic RNA *in situ* hybridization: the detection of low copy nbumber mRNA molecules in bone marrow cells of multiple myeloma patients. In: Monoclonal gamapathies. II. Clinical significance and basic mechanisms. Radl,

J. and Van Camp, B. (Eds.) 1989, p. 113-116, Eurage.

M.A. Versnel, M.J. Bouts, H.C. Hoogsteden, Th.H. van der Kwast, M. Delahaye and A. Hagemeijer. Establishement of human malignant mesothelioma cell lines. Int. J. Cancer 44, 256-260, 1989.

M.A. Versnel, H.C. Hoogsteden, A. Hagemeijer, M.J. Bouts, Th.H. van der Kwast, M. Delahaye, G. Schaart and F.C.S. Ramaekers. Characterization of three human malignant mesothelioma cell lines. Cancer Genet. Cytogenet., in press.

T. van Gelder, H.C. Hoogsteden, M.A. Versnel, J.P. Vandenbroucke and H.T. Planteydt. Malignant pleural mesothelioma in the Southwestern part of The Netherlands. Eur. Resp. J., in press.

T. van Gelder, H.C. Hoogsteden, M.A. Versnel, J.P. Vandenbroucke and H.T. Planteydt. Malignant peritoneal mesothelioma: a series of 19 cases. Digestion, in press.

A. Hagemeijer, M.A. Versnel, E. van Drunen, M. Moret, M.J. Bouts, Th.H. van der Kwast and H.C. Hoogsteden. Cytogenetic analysis of malignant mesothelioma. Cancer Genet. Cytogenet, in press.

DANKWOORD

Vele mensen hebben op enigerlei wijze een bijdrage geleverd aan de totstandkoming van dit proefschrift. Slechts enkelen daarvan kan ik hier noemen.

Allereerst wil ik mijn ouders danken voor de kansen die zij mij hebben geboden. Jullie hebben mij altijd gesteund en gestimuleerd.

Rob Benner, jou bedank ik hartelijk voor zijn enthousiaste begeleiding van het onderzoek, het in mij gestelde vertrouwen en de vrijheid die mij gegund werd. Anne Hagemeijer, jouw betrokkenheid bij het mesothelioomonderzoek, de plezierige en opbouwende wijze waarop jij mijn manuscripten besprak en het feit dat je mijn promotor wilde zijn, heb ik bijzonder op prijs gesteld.

De leden van de promotiecommissie professor Bootsma en Joop van Zoelen bedank ik voor hun waardevolle suggesties en voor het beoordelen van het manuscript voor dit proefschrift.

Henk Hoogsteden, jij gaf de eerste aanzet tot het mesothelioomonderzoek in Rotterdam. Jouw steun en betrokkenheid daarbij heb ik bijzonder gewaardeerd. Professor Hilvering wil ik danken voor de bekendheid die hij aan ons projekt heeft gegeven onder de longartsen in de regio's Rotterdam en Vlissingen. Deze longartsen werkten mee aan het projekt en leverden het patiëntenmateriaal dat onmisbaar was voor dit onderzoek. De medewerkers van de afdeling Pathologie van het Ziekenhuis Dijkzigt, dank ik voor hun bijdragen in verschillende fasen van het onderzoek. Theo van der Kwast en Mick Delahaye dank ik voor de grote interesse die zij stelden in mijn onderzoek en hun bijdrage aan de diagnostiek van de mesotheliomen. Dr. H.T. Planteijdt wil ik ook noemen in verband met de diagnostiek, bovendien heb ik zijn belangstelling voor ons projekt bijzonder gewaardeerd.

From the Ludwig Institute for Cancer Research, Uppsala, I want to thank Carl-Henrik Heldin for his hospitality, advice and generosity with ^{125}I-PDGF, Lena Claesson-Welsh for the gift of PDGF receptor probes, en Annet Hammacher voor de prettige samenwerking gedurende mijn verblijf te Uppsala.

Frans Ramaekers en Gert Schaart van de afdeling Pathologie van de Katholieke Universiteit Nijmegen dank ik voor de goede samenwerking op het gebied van de keratines.

Monique Bouts, jouw enthousiasme en grote inzet waarmee je experimenten hebt uitgevoerd en niet in de laatste plaats de attenties op onverwachte momenten heb ik bijzonder gewaardeerd. Zij droegen zeker bij tot de plezierige werksfeer en ik ben blij dat je mij als paranimf terzijde zult staan.

Van het Cytogenetica lab noem ik Bep Smit, Marian Moret, Ellen Schoenmaker en Elsa Franken. Ik dank jullie voor de met zorg uitgevoerde chromosoomanalyses. Gemma Vredeveldt werkte als doctoraal studente enthousiast aan het projekt mee. Tom Vermetten en Rein Smid zorgden voor de bestellingen en Piet Hartwijk verleende bijstand als er iets kapot gegaan was. De steriliteit van het glaswerk en de zorg voor de koffie vormden de onmisbare bijdrage van mevrouw Godijn, Jopie Bolman en Elly Hoffman. Gea Stout, Jacqueline de Goey en Geertje de Korte wil ik danken voor het typewerk dat nodig was voor het verwerken van onderzoeksresultaten tot manuscripten. En Tar van Os voor de secure wijze waarop hij de foto's en dia's maakte. Geertje en Tar, jullie verleenden onmisbare steun bij de afronding van mijn proefschrift.

Ook de andere medewerkers van de afdeling wil ik danken voor de plezierige werksfeer. Dit geldt met name voor de bewoners van het diagnostisch lab waar ik met plezier gewerkt heb. Jacques van Dongen, Herbert Hooijkaas en Ingrid Wolvers-Tettero wil ik danken voor hun hulp in de beginfase van het projekt. Henk Adriaansen, het was prettig om met jou een studeerkamer en daardoor ook de nodige ups en downs te delen. Bovendien stel ik het op prijs dat je mijn paranimf bent.

Hans, jou wil ik danken voor de steun vanaf het eerste moment dat ik naar Rotterdam ging. Samen met Frank slaagde je erin ook de afgelopen maanden mijn aandacht op andere zaken dan dit proefschrift te richten.

ABBREVIATIONS

bcr	breakpoint cluster region
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
cpm	counts per minute
c-*sis*	cellular homologue of SSV
der	derivative
del	deletion
dup	duplication
dm	double minute
DM	direct method
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EM	electron microscopy
EMA	epithelial membrane antigen
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTG	G banding by trypsin giemsa
HC	hydrocortisone
HMFG	human milk fat globule
HSR	homogeneously staining region
i	isochromosome
IFP	intermediate filament protein
inv	inversion
ISCN	international system for cytogenetic nomenclature
kb	kilo base
kD	kilo dalton
MAbs.	monoclonal antibodies
mar	marker
Me-	mesothelioma

Mero-	mesothelioma Rotterdam (cell line)
MM	malignant mesothelioma
mRNA	messenger ribonucleic acid
p	short arm chromosome
PAS-d	periodic acid Schiff-diastase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
q	long arm chromosome
QFQ	Q banding by fluorescence after quinacrine
RFA	R banding by fluorescence after acridine orange
RNA	ribonucleic acid
s.c.	subcutaneous
SSC	standard saline citrate
SSV	simian sarcoma virus
t	translocation
TEM	transmission electron microscopy
ter	terminal segment of chromosome arm
TGF	transforming growth factor