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Immunoreactivity for c-fos and c-myc protein with the monoclonal antibodies 14E10 and 6E10 in malignant mesothelioma and non-neoplastic mesothelium of the pleura

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Summary. We studied immunoreactivity for c-fos protein and c-myc protein in malignant mesothelioma (36 cases) and non-neoplastic pleural mesothelium (45 cases) using the murine monoclonal antibodies 14E10 and 6E10. All malignant mesotheliomas and cases with non-neoplastic mesothelium exhibited not only nuclear but also cytoplasmic immunoreactivity for c-fos and c-myc protein in the majority of mesothelial cells. There was no statistically significant difference between the various mesothelioma subtypes or between neoplastic and non-neoplastic mesothelium for c-fos protein immunoreactivity ($p>0.05$). There was statistically significant difference between neoplastic and non-neoplastic mesothelium for c-myc protein immunoreactivity ($p<0.01$).

We conclude that immunoreactivity for c-fos and c-myc protein is present in both non-neoplastic and neoplastic mesothelium, but that a higher proportion of neoplastic mesothelial cells are immunoreactive for c-myc protein when compared with non-neoplastic mesothelium.

Key words: Fos, Myc, Oncogene, Mesothelioma, Pleura, Immunohistochemistry

Introduction

The v-myc gene was first identified as the transforming gene of the avian leukosis virus MC29 (Sheiness et al., 1978). The fos gene was identified as the transforming sequence of the FBR Moloney sarcoma virus inducing osteogenic sarcomas in rodents (Vingron et al., 1988). Expression of the homologous cellular c-myc and c-fos protooncogene has been found in various normal tissues (Müller et al., 1983; Slamon et al., 1984; De Togni et al., 1988; Loke et al., 1988; Basset-Seguin et al., 1990; Klimpfinger et al., 1990). Both c-fos and c-myc protein are DNA binding proteins that can bind certain DNA sequences and enhance transcriptional activation of multiple genes (Chiu et al., 1988). Abnormalities of the c-myc gene, such as amplification, translocation and deregulation have been described in lung cancer, Burkitt lymphoma and colonic cancer resulting in overexpression of the c-myc protein (Erisman et al., 1985; Cory, 1986; Wong et al., 1986; Williams et al., 1990). Malignant mesotheliomas are tumours originating in the serosal lining of the pleural cavities. The molecular alterations leading to mesothelioma are still unclear. The majority of human pleural mesotheliomas are associated with asbestos exposure (Antman, 1980). Recently, Cicala and coworkers (1993) described the induction of mesotheliomas by SV40 virus in hamsters, indicating a viral cofactor in the genesis of malignant mesothelioma. In addition, an RNA virus, the MC29 avian leukosis virus, has been found to induce mesotheliomas in 35% of chickens infected (Chabot et al., 1970).

Because these experiments seem to suggest a possible viral cofactor for inducing mesothelioma, we decided to investigate the presence of c-fos and c-myc immunoreactivity in malignant mesothelioma, since both oncogenes are known to be present in some viruses, and to compare this with non-neoplastic mesothelium (Chabot et al., 1970; Cicala et al., 1993).

Materials and methods

A total of 36 paraffin-embedded mesothelioma tissue specimens, comprising 25 epithelial mesotheliomas, 8 mixed mesotheliomas and 3 mesenchymal mesotheliomas, were included in the study, together with 45 paraffin-embedded tissue specimens of pleura or pleural exudates with non-neoplastic mesothelium, including 25 specimens with signs of hyperplasia.

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Asbestos exposure was documented in all mesothelioma cases. Additional histochemical stainings were performed, including PAS, PAS after diastase treatment, alcian blue and alcian blue after hyaluronidase treatment. Immunohistochemical stainings for carcinoembryonic antigen (CEA), cytokeratin, vimentin and epithelial membrane antigen (EMA) were also performed employing standard PAP immunohistochemical procedures. All mesotheliomas exhibited alcian blue hyaluronidase-sensitive positivity and no neutral mucins were found. Immunohistochemically, all were immunoreactive for cytokeratin, vimentin and epithelial membrane antigen (EMA). No CEA positivity was found. No neutral mucins were found with the PAS after diastase digestion.

Five micrometer thick sections were cut, dewaxed in xylene followed by rehydration. The sections were consecutively treated for 30 minutes with:

1) primary antibody. Anti-c-fos protein mouse monoclonal antibody 14E10, isotype IgG_1 (supernatant of 14E10 mouse-human hybridoma cell-line, NIH) diluted 1/5 in PBS supplemented with 1% bovine serum albumin at room temperature. This monoclonal antibody is directed against a synthetic tridecapeptide derived from the aminoterminal region (aminoacids 4-17: SGFNADYEASSRC) of the human c-fos protein (De Togni et al., 1988). It recognizes not only the fos protein complex with the cellular 39 kD protein, but also the modified v-fos forms of the mouse, rat and human fos proteins in an immunoprecipitation assay and in immunohistochemical techniques (De Togni et al., 1988) anti-c-myc protein mouse monoclonal antibody 6E10, isotype IgG_1 (Cambridge Research Biochemical, Cambridge U.K.), diluted 1/100 in PBS supplemented with 1% bovine serum albumin at room temperature. This monoclonal antibody is directed against a synthetic peptide (D-peptide) comprising the amino acid residues 171-188 (CSTSSLYLQDLSAAASEC) of the p62 c-myc protein (Evan et al., 1985).

The specificity of both antibodies in immunohistochemical reactions has been amply described (Jack et al., 1986; Stewart et al., 1986; Sikora et al., 1987; De Togni et al., 1988; Polacarz et al., 1989; Sasano et al., 1992).

2) rabbit anti-mouse polyclonal antibody (Dako, Glostrup, Denmark), diluted 1:20 in PBS with 1% normal human serum.

3) horseradish peroxidase mouse polyclonal anti-horseradish peroxidase complex diluted 1:250 in PBS (Dako, Glostrup, Denmark).

Steps two and three were repeated for 10 minutes to enhance the reaction.

Peroxidase was revealed by incubating the sections with a solution containing 3,3'-diaminobenzidinetetrahydrochloride in 20 ml TRIS buffer, pH 7.6, containing 2% hydrogen peroxide.

The specificity of the immunohistochemical reactions was controlled as follows: 1) by omitting the first antibody; 2) by substituting the anti-c-fos or anti-c-myc antibody for an unrelated monoclonal antibody of the same isotype IgG_1 in the same concentration but directed against an unrelated antibody (monoclonal mouse anti-human IgM antibody, isotype IgG_1, Dako, Denmark).

Sections of skin (Basset-Seguin et al., 1990) and colonic mucosa (Royds et al., 1992) were used as positive controls for c-fos protein and c-myc protein.

The chi-square test was used for statistical analysis.

Results

In mesotheliomas, staining of both cytoplasm and nucleus was found in all cases with the 14E10 anti-c-fos antibody and the 6E10 anti-c-myc monoclonal antibody (Figs. 1, 2). Not all tumour cells were immunoreactive. Nuclear staining was in a fine granular diffuse fashion with appearance of the nucleolar regions. Cytoplasmic staining was homogeneous and diffuse. In two malignant mesotheliomas, one epithelial and one mixed type, there was nuclear and cytoplasmic immunoreactivity for c-fos protein in 50-75% of the neoplastic cells (Table 1). In all other mesotheliomas and

Table 1. Immunohistochemical staining of c-fos protein neoplastic and non-neoplastic mesothelial tissues. Absolute numbers are given (percentages between brackets).

MESOTHELIOMA TYPE	% OF POSITIVE CELLS			
	5-25	25-50	50-75	>75
Epithelial			1 (4%)	24 (96%)
Mixed			1 (1%)	7 (88%)
Mesenchymal				3 (100%)
All mesotheliomas			2 (6%)	34 (96%)
Normal mesothelium			3 (15%)	17 (85%)
Reactive mesothelium	3 (12%)		3 (12%)	19 (76%)
All non-neoplastic mesothelium	3 (6%)		6 (13%)	36 (80%)

Table 2. Immunohistochemical staining of c-myc protein neoplastic and non-neoplastic mesothelial tissues. Absolute numbers are given (percentages between brackets).

MESOTHELIOMA TYPE	% OF POSITIVE CELLS			
	5-25	25-50	50-75	>75
Epithelial		2 (8%)	5 (20%)	18 (72%)
Mixed		1 (12%)	2 (25%)	5 (63%)
Mesenchymal		1 (33%)		2 (67%)
All mesotheliomas		4 (12%)	7 (19%)	25 (69%)
Normal mesothelium	3 (15%)	8 (40%)	5 (25%)	4 (20%)
Reactive mesothelium	1 (4%)	7 (28%)	9 (36%)	8 (32%)
All non-neoplastic mesothelium	4 (9%)	15 (33%)	14 (31%)	12 (27%)

in cases with non-neoplastic mesothelium more than 75% of all neoplastic cells were immunoreactive for c-fos protein. Statistical analysis did not reveal a difference between neoplastic and non-neoplastic mesothelium or between the various mesothelioma subtypes ($p>0.05$), as determined by the Chi-square test. The majority of malignant mesotheliomas were immunoreactive for c-myc protein in more than 50% of the neoplastic cells (Table 2). Non-neoplastic mesothelium displayed a broader range of immunoreactivity ranging from 5 to 75% positive mesothelial cells. There was a statistically significant difference between non-neoplastic mesothelium and neoplastic mesothelium ($p<0.01$) as determined by the Chi-square test. Positive control sections showed moderate to strong nuclear and cytoplasmic immunoreactivity in keratinocytes for c-fos

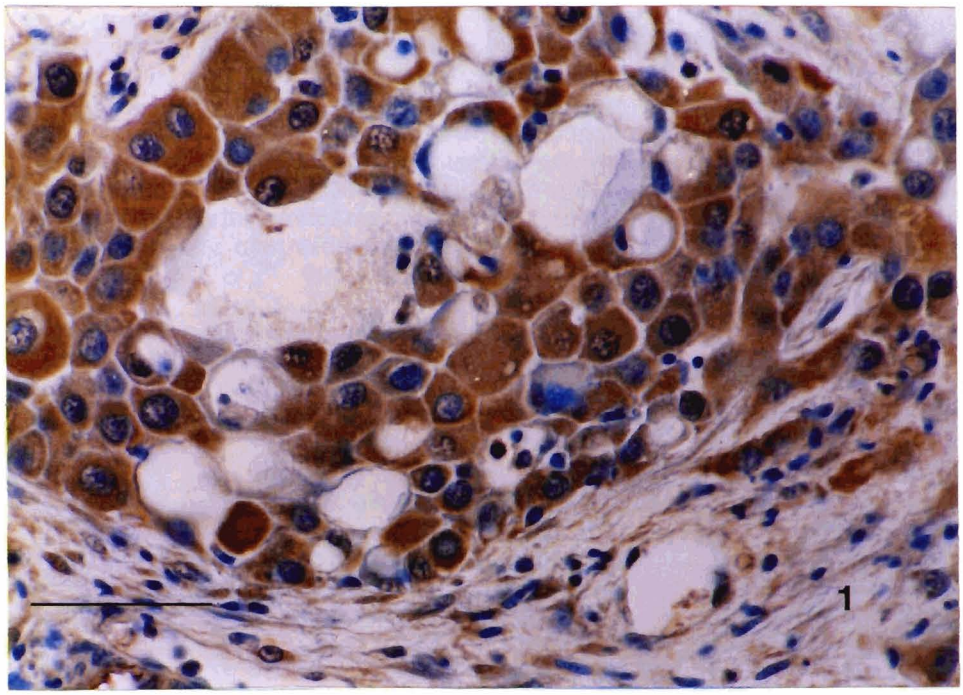

Fig. 1. Nuclear and cytoplasmic immunoreactivity for c-fos protein in an epithelial mesothelioma. Bar= 200 µm. x 100

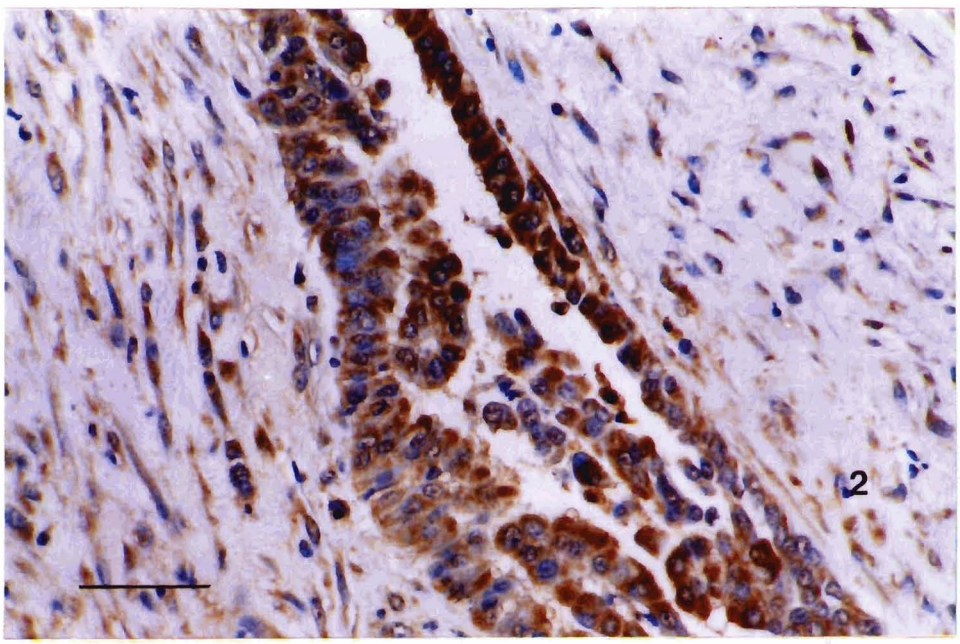

Fig. 2. Nuclear and cytoplasmic immunoreactivity for c-myc protein in an epithelial mesothelioma. Bar= 200 µm. x 100

protein and for c-myc protein in enterocytes.

Discussion

In our study we found immunoreactivity for c-fos and c-myc protein in both neoplastic and non-neoplastic mesothelium. One may assume that both nuclear proteins can be induced in mesothelium by growth factors such as EGF and PDGF by a similar mechanism to that described in 3T3 fibroblasts (Müller et al., 1984). Malignant mesothelioma and non-neoplastic mesothelium are known to express growth factor receptors for EGF (Ramael et al., 1991) and PDGF (Ramael et al., 1992). From this point of view, the observed immunoreactivity for c-fos and c-myc would only be the consequence of growth factor stimulation and would not be the primary event in the genesis of malignant mesothelioma. However, there was a significant difference between neoplastic and non-neoplastic mesothelium for c-myc protein immunoreactivity in contrast to c-fos protein. This raises the question as to the role of suppressor genes in malignant mesothelioma, such as retinoblastoma protein (Rb p107), as the latter can compound with c-myc protein. The N-terminal domain of c-myc mediates binding to the retinoblastoma gene product (Rustgi et al., 1991). Normal p107 Rb protein can suppress c-myc transcription by interacting with the TGF-ß control element (TCE) sequence in the c-myc promotor (Pietenpol et al., 1991). Abnormalities in the retinoblastoma gene would result in loss of binding to the TCE sequence of the c-myc promotor resulting in depression of the c-myc gene, consequently followed by an increase of c-myc gene product. This hypothesis is corroborated by the fact that most malignant mesotheliomas express an abnormal Rb protein, in contrast to non-neoplastic mesothelium which express a normal Rb protein (Ramael et al., 1993). This may be an explanation of the observed difference for c-myc immunoreactivity between neoplastic and non-neoplastic mesothelium. There was no statistically significant difference for c-fos immunoreactivity between neoplastic and non-neoplastic mesothelium, suggesting that fos immunoreactivity in mesothelial tissues might be merely a differentiation-related event, as described for the F8 teratocarcinoma cell line (Müller and Wagner, 1984). Our results are comparable to the reported expression of c-fos in benign and malignant breast tissue where in both categories there was expression with a significant overlap between benign and malignant tissue (Walker and Cowl, 1991).

We did not only find nuclear immunoreactivity for c-fos and c-myc protein in all mesotheliomas, but also observed cytoplasmic immunoreactivity, although both c-fos and c-myc protein are known to be nucleus-associated proteins (Eisenmann et al., 1985). It is possible that cytoplasmic immunoreactivity for nuclear proteins, including c-myc protein, is a fixation-related artifact, as fixation times longer than 60 to 120 minutes seem to result in cytoplasmic immunoreactivity (Loke et al., 1988). The c-fos protein undergoes extensive post-translational modifications in the cytoplasm converting the initial p55 protein into a series of distinct products of 57, 60 and 62 kD before the protein is translocated to the nucleus (Franza et al., 1987). These findings explain the presence of cytoplasmic immunoreactivity for c-fos protein in our study, as the 6E10 monoclonal antibody recognizes not only the fos protein complex with the cellular 39 kD protein, but also the modified v-fos forms of the mouse, rat and human fos proteins.

In this study we described immunoreactivity for c-fos and c-myc protein in both malignant mesothelioma and in non-neoplastic mesothelium. There was no significant difference between neoplastic and non-neoplastic mesothelium for c-fos immunoreactivity. There was a significant difference between neoplastic and non-neoplastic mesothelium for c-myc protein, indicating that c-myc immunoreactivity might be the expression of hitherto unknown abnormalities in malignant mesothelioma.

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