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ARTICLE

Effect of Ribonuclease A and Deoxyribonuclease I on Immunostaining of Ki-67 in Cultured Melanoma Cells

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Immunostaining of the cell cycle-associated Ki-67 antigen was studied, using the Ki-67-specific MIB-1 monoclonal antibody on slides prepared by cytocentrifugation of cultured A375 melanoma cells. Immunomorphological analysis of the Ki-67 immunostaining pattern of both nuclear and nucleolar locations was carried out following pretreatment of the slides including ribonuclease and deoxyribonuclease pre-digestion of the cells. Immunostaining of nucleolar Ki-67 was reduced by ribonuclease pre-digestion, but was not altered by deoxyribonuclease pre-treatment. Ribonuclease did not reduce the staining intensity of Ki-67 in the nuclear matrix, but the intensity decreased after deoxyribonuclease pre-digestion. We suggest that the Ki-67 molecule may play an important role in ensuring contact between nuclear DNA and nucleolar RNA during transcriptional processes in cell proliferation. (Pathology Oncology Research Vol 2, No1–2, 63–65, 1996)

Key words: Key words: Ki-67, immunocytochemistry, enzymatic pre-digestion, proliferating cells

Introduction

An important part of the histopathologic study of malignant cells is the assessment of their proliferative potential.[4,19] Immunohistochemical analysis of nuclear antigens associated with cell proliferation makes possible cellular kinetic studies in correlation with the architectural organization of proliferating cells.[12,17] For routine use, the new MIB-1 monoclonal antibody[1,7,8,11] is the best currently available proliferation marker.[2,6,13,14] It reacts with the proliferation-associated nuclear antigen, previously defined by the antibody Ki-67.[9] The nuclear Ki-67 antigen is detectable exclusively in proliferating cells, that is in G1, S, G2 and M phases, but not in G0.[3] The molecular and genetic basis of the Ki-67 antigen and that of the immunoreactivity of the MIB-1 monoclonal antibody is well documented[5,8,9,16], but the function of the Ki-67 protein is unknown. It may be an absolute requirement for maintaining cell proliferation,[16] it may play a role in the breakdown of the nuclear envelope prior to mitosis[3], or it may represent a major structural protein during mitosis.[15]

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We observed recently that, using the Ki-67-specific MIB-1 monoclonal antibody on routinely-fixed, paraffin embedded tissue sections, pre-heated in a microwave oven, immunostaining of nucleolar Ki-67 was reduced by ribonuclease (RNase) pre-digestion; however, it was not significantly reduced by deoxyribonuclease (DNase) treatment.[18]

The aim of the present study was to evaluate the action of RNase and DNase, respectively on Ki-67 immunostaining in samples of cultured malignant cells.

Materials and Methods

Cell line

A375 cells (malignant melanoma-derived cell line, courtesy of Dr. Felix Montero, Marseille, France) were cultured under standard conditions in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum and antibiotics. Cytocentrifuged (1000 rpm for 10 min, Shandon Cytospin 3, UK) A375 cells were fixed in cold (4°C) acetone for 10 min followed by a supplementary fixation in a mixture of concentrated acetone and methanol (v/v) at -20°C for 5 min; the slides were stored at -80°C.

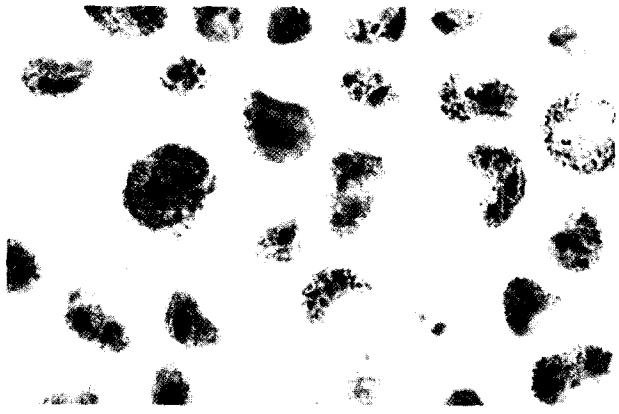

Figure 1. Immunostaining of Ki-67, using the monoclonal MIB-1 antibody and the streptavidin-biotin-peroxidase method, of cultured A375 melanoma cell line. Without enzymatic pre-treatment, the staining is nuclear and nucleolar. The cells in mitosis are also positive. DAB, hematoxylin counterstain, 320 x.

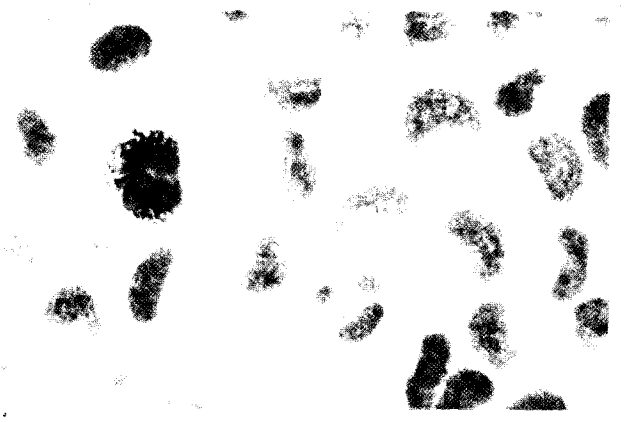

Figure 2. Immunostaining of Ki-67, using the monodonal MIB-1 antibody and streptavidin-biotin-peroxidase method, on the cultured A375 melanoma cell line. Following RNase predigestion (0.5 U/ml), the nuclear staining and that of mitotic cells is unaltered, however, nudeoli are negative. DAB, hematoxylin counterstain, 320 x.

Enzymatic digestion

For RNase and DNase digestion of the mounted cells, the slides were incubated with several concentrations of RNase A (EC 3.1.27.5, 90 U/mg, Fluka, Buchs, Switzerland) and DNase I (EC 3.1.21.1, 3000 U/mg, Fluka), respectively, for 20 min at 37°C. After enzymatic digestion the slides were washed with pH 7.6 Tris-buffered saline (TBS).

Immunocytochemistry

The slides were incubated at room temperature for 60 min with the Ki-67-specific MIB-1 monoclonal antibody (Immunotech, Marseille, France) at a dilution of 1/100 in TBS. Immunocytochemical staining was carried out according to the streptavidin-biotin-peroxidase technique with H_2O_2/diaminobenzidine (DAB) development, using the Immunotech Universal Kit. Slides were also tested without enzymatic treatment. Immunostaining with ascites fluid from non-immunized mice served as a negative control.

***Table 1.* Topological distribution and intensity of Ki-67 immunostaining in cultured A375 melanoma cells using the MIB-1 antibody and different enzymatic pre-digestions**

Pretreatment	*Immunostaining intensity*		
	Nucleoli	*Nuclear matrix*	*Mitotic cells*
None	+/++	+/++	++
RNase 0.5 U/ml	−/+	+/++	++
DNase 100 U/ml	+/++	−/+	−

− negative; + weak, ++ strong; slash indicates heterogeneity of staining intensity at cellular level

Results

The immunocytochemical results are summarized in *Table 1*. Without enzymatic pre-treatment, the monoclonal MIB-1 antibody reacted with about 90% of the cultured melanoma cells (*Fig. 1*). Mitotic cells were usually positive. The immunostaining reaction appeared as brown staining of the nuclei with significant variation in color intensity and staining pattern. This heterogeneity reflected heterogeneous distribution of the Ki-67 antigen. We observed cells with nucleolar staining only, other cells with predominantly nucleolar staining and staining of a nuclear component; in yet other cells the nuclear matrix stained strongly. In mitotic cells, the immunostaining was cytoplasmic with perichromosomal intensifcation.

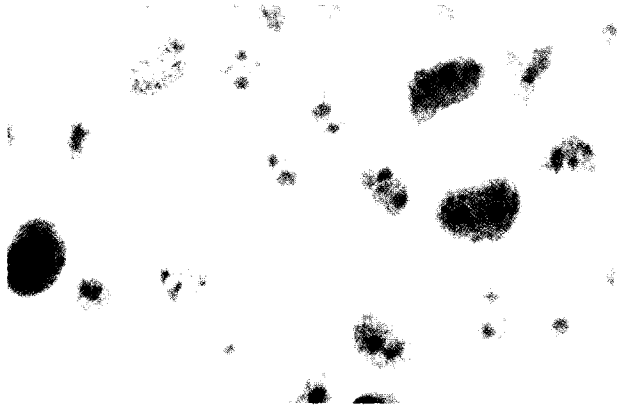

Figure 3. Immunostaining of Ki-67, using the monoclonal MIB-1 antibody and streptavidin-biotin-peroxidase method, of cultured A375 melanoma cell line. Following DNase pre-treatment (100 U/ml), a significant decrease of nuclear matrix staining was observed; mitotic cells were negative, however, nucleoli were positive. DAB, hematoxylin counterstain, 320 x.

After RNase pre-treatment, we observed positive mitotic cells and unaltered nuclear staining; however, the nucleolar staining was lost and/or reduced (*Fig.2*).

The DNase pre-treatment strongly reduced the intensity of nuclear staining, and we did not observe immunoreactivity in mitotic cells (*Fig.3*). The nucleolar staining pattern was not altered by DNase treatment. Both RNase and DNase activities were concentration-dependent (*Table 2*).

Discussion

In this study, cultured melanoma cells were exposed to RNase and DNase pre-digestion, respectively, prior to immunostaining for Ki-67 with monoclonal antibody MIB-1. The results confirmed our previously reported findings obtained on fixed-embedded tissue sections, that RNase predigestion reduces immunostaining of nucleolar Ki-67.[18] However, the DNase sensitivity of the Ki-67 antigen was not observed in fixed-embedded tissues, but in this study, this enzyme significantly reduced Ki-67 immunostaining of cultured cells, in particular in mitotic cells. Gerdes et al[5] reported that Ki-67 immunostaining in frozen sections was resistant to both RNase and DNase; they used lower enzyme

***Table 2.* Effect of RNase and DNase pre-digestion on Ki-67 immunostaining in cultured A375 melanoma cells using the MIB-1 antibody and several enzyme concentrations**

RNase (U/ml)	*Nucleolar staining intensity*	*DNase (U/ml)*	*Nuclear matrix staining intensity*
0.001	+/++	10	+/++
0.01	+/++	20	+/++
0.1	+	50	−/+
0.5	−/+	100	−/+
1.0	−	200	−

− negative; + weak, ++ strong, slash indicates heterogeneity of staining intensity

concentrations than we did. Lopez et al[10] reported DNase sensitivity of the Ki-67 antigen studied by flow cytometry, but they did not observe significant reduction of Ki-67 mean fluorescence in RNase pre-treated cells. Our results lead us to conclude that the Ki-67 located in the nucleoli is associated there with nucleolar RNA, whereas in the nuclear matrix, it is strongly associated with DNA. It may play a role in transcriptional processes, probably as a structural protein, perhaps by mediating between nuclear DNA and nucleolar RNA.

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