Expression of HMGI(Y) Proteins in Squamous Intraepithelial and Invasive Lesions of the Uterine Cervix

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ABSTRACT

The expression of nuclear proteins high mobility group (HMG) I and HMGY was investigated in intraepithelial and invasive lesions of the uterine cervix. Human carcinoma cell lines C-41, ME-180, and CaSkii were used for testing protein expression in neoplastic cells from the cervix. Morphological grading of the dysplasias (CIN 1, CIN 2, and CIN 3) and invasive carcinomas from formalin-fixed paraffin-embedded samples parallels the degree of nuclear immunostaining obtained using a polyclonal antibody raised against the amino-terminal region of HMGI(Y) proteins. The immunostaining obtained with HMGI(Y) antibody was compared with that observed using the antibody Ki-67, and the results were similar. We suggest the use of HMGI(Y) antibody in clinical oncology as a useful marker of intraepithelial lesions and invasive carcinomas.

INTRODUCTION

Since the discovery of HMG

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I proteins as components of the chromatin of mammalian cells (1–3), the attention of researchers has been attracted by the fact that high levels of expression of these proteins are observable in neoplastic transformed cells by comparison with their normal counterparts, in which HMG proteins are absent or detectable at very low levels (3, 4). The group is formed by three polypeptides named HMGI (which gives the name to the entire group), HMGY, and HMGI-C (1–5), all constituted by about 100 amino acid residues and showing a preferential binding to A+T-rich DNA sequences (6–11), mainly due to a repeating stretch of positive amino acid residues contained three times and highly conserved in the sequence of the three proteins (12, 13). Proteins HMGI and HMGY derive from the same gene by alternative splicing (12) and are currently indicated as HMGI(Y) proteins. However, HMGI-C is the product of a different gene that expresses a protein having about 50% sequence homology with HMGI(Y) (13, 14).

It has been demonstrated that HMGI(Y) proteins participate as structural factors in protein complexes that stimulate the transcription of genes such as IFN-ß, tumor necrosis factor ß, and interleukin 2 receptor α chain (15–19). On the other hand, it has also been reported that chromosomal aberrations in the region 12q14–15 involve HMGI-C gene rearrangements that correlate to the onset of benign solid tumors such as lipomas and pulmonary chondroid hamartomas (20–22), whereas constitutional knockout of the Hmgic-1 mouse gene induces the pygmy phenotype (23).

Finding new markers that could aid in the early detection of cancer, in the evaluation of the metastatic potentiality of cancerous cells, and in therapy monitoring is a general aim of oncological research. To this end, tumor markers such as carcinoembryonic antigen, proliferating cell nuclear antigen, nuclear proliferative antigen Ki-67, and AgNOR proteins are widely used (24, 25). Each of the suggested markers refers to an antigen involved in a different aspect of the cellular activity; therefore, a higher reliability of the assessment of cell proliferation and/or neoplastic transformation could be achieved by the use of more than one marker. Due to the high level of expression of HMGI proteins found in both transformed cell lines and tumors having different origins (4, 5, 26, 27), we and others have suggested HMGI(Y) proteins as new markers of the transformed cellular phenotype and used them as a progression marker for prostate cancer metastasis (28, 29) and malignant phenotype assessment of both human thyroid and colorectal neoplasias (30, 31).

As a further extension of the study of the expression of HMGI proteins in tumor tissues, here we report the use of an anti-HMGI(Y) antibody as a diagnostic aid in the grading of intraepithelial and invasive lesions of the uterine cervix. Immunohistochemical results were compared on one hand with the morphological diagnoses and, on the other hand, with parallel immunohistochemical data obtained using the Ki-67 proliferating marker. Our results allow us to suggest anti-HMGI(Y) antibody as a helpful aid in clinical oncology.

MATERIALS AND METHODS

Cell Cultures and Tissues. The three human cervix carcinoma cell lines (C4-I, ME-180, and CaSkii) used in this study for testing HMGI(Y) protein expression were purchased from American Type Culture Collection (Rockville, MD) and grown in DMEM containing 10% FCS. Epithelial rat thyroid FRTL-5 cells were cultured as reported previously (3). Tumors from Lewis lung carcinoma cells were induced in mice as described previously (26).

Normal and pathological specimens from human cervix tissue for immunohistochemical analyses were obtained from formalin-fixed paraffin-embedded samples of the tissue file of the Istituto di Anatomia ed Istologia Patologica of the University of Trieste.

Antibodies. A polyclonal antibody was raised in rabbits using the synthetic peptide SSSKSSQPLASKQ specific for the HMGI(Y) proteins and affinity purified. The antibody was tested for activity by Western blot analysis using high-performance liquid chromatography-purified samples of recombinant HMGI(Y) proteins. Ki-67 monoclonal antibody was purchased from DAKO.

Western and Northern Blot Analyses. Total HMG proteins were selectively extracted by 5% (v/v) perchloric acid from cultured cells and tumors and recovered by precipitation with acetone as reported previously (3, 26). Resolution of the two electrophoretic bands due to proteins HMGI and HMGY was achieved by polyacrylamide (15%) SDS/Trit/Tricine gel electrophoresis (5) run for 17 h at 200 V. For Western analysis, gels were soaked in 750 mM Tris and proteins were recovered by precipitation with acetone as reported previously (3, 26). Resol

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3 The abbreviations used are: HMG, high mobility group; CIN, cervical intraepithelial neoplasia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAB, 3,3′-diaminobenzidine.
Immunohistochemistry. Cultured cell suspension (200 μl) was centrifuged (Cytospin) for 5 min at 500 rpm, obtaining a 5-mm diameter area on a slide. Cells were then fixed with acetone/methanol (1:1, v/v) for 10 min at −20°C and soaked in PBS.

The following smears and formalin-fixed paraffin-embedded cases of normal and pathological tissues were selected: (a) 3 smears from normal epithelia; (b) 28 normal cervixes; (c) 25 low-grade lesions (CIN 1); (d) 10 moderate-grade lesions (CIN 2); (e) 25 high-grade lesions (CIN 3); and (f) 27 invasive squamous carcinomas. About 3-μm sections were cut with a microtome and dried overnight at 37°C on a slide for immunohistochemical analysis. Samples were deparaffinized by xylene at room temperature for 30 min and washed with graded ethanol/water mixtures (100, 90, 70, and 50% ethanol) before washing with tap water and then with distilled water. Samples were then put in the microwave for 30 min and subsequently left at room temperature for 30 min. After washing with tap water and distilled water, samples were soaked in PBS.

Both cellular and tissue specimens were treated with 3% hydrogen peroxide for 5 min and washed as described above before immunoperoxidase staining. Samples were incubated in normal horse serum for 10 min and then treated for 60 min at room temperature with the antibody diluted 1:100 or 1:200 in PBS. The slides were washed in PBS three times for 5 min and then incubated with biotinylated universal secondary horse antibody (Vector Laboratories). After washing, slides were incubated for 10 min in streptavidin/peroxidase complex. Immunostaining was performed by incubation of the slides in DAB (Abbott Laboratories) solution or aminoethylcarbazole (Biogenex). Slides were then washed with water and counterstained with hematoxylin for 30 s. Finally, slides from tissues were washed, dehydrated, and mounted with permanent mounting medium (Entellam; MERCK); cell culture slides were directly mounted in aqueous mounting medium (Glycergelet; DAKO). Micrographs were taken on Kodak Ektachrome film with a photomicroscope (Leitz).

RESULTS

**HMG1 Gene Expression in Cell Lines Obtained from Squamous Carcinomas of the Uterine Cervix.** The expression of the HMG1(Y) gene in carcinomas from the uterine cervix was tested by both Northern and Western blot analyses. About 20 μg of total RNA were loaded in each slot; GAPDH was used as an internal control for uniform RNA loading. Western blot analysis of HMG1(Y) proteins extracted from the same cell lines. About 10 μg of total extracted protein were loaded in each slot, and equal loading was assured by checking the amount of histone H1 coextracted with HMG1(Y) proteins and stained with Coomasie Blue. Lanes 1, Lewis lung carcinoma cells, positive control; Lanes 2, FRTL-5 cell line, negative control; Lanes 3, C-41 cervix carcinoma cell line; Lanes 4, CaSki cervix carcinoma cell line; Lanes 5, ME-180 cervix carcinoma cell line. Results show that HMG1(Y) proteins are expressed in all three cervix carcinoma cell lines.

Comparison between Immunostaining of Nuclear Antigens Using HMG1(Y) Antibody and Ki-67 Antibody. The antibody Ki-67 is frequently used in the assessment of proliferation activity. Although it recognizes a nuclear antigen whose function is still unknown (33), positive staining with this antibody is considered of significant im-
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Fig. 2. Immunohistochemical analysis of HMG(Y) protein expression in normal squamous superficial epithelial cells from cervical smears (A1 and A2) and in cervix carcinoma cell lines C-4I (B), ME-180 (C), and CaSki (D). Objective magnification: ×100, A2 and B-D; ×40, A1. Cells were immunostained with streptavidin/peroxidase/aminoethylcarbazole and counterstained with hematoxylin. No immunostaining was observed in normal cells (A1 and A2), whereas strong nuclear positivity was detected in carcinoma cells (B-D).

The importance in surgical pathology, because antigen recognition correlates with the proliferation degree of the cells (24, 33–37). Therefore, we thought that it could be of some interest to compare the immunostaining of the same tissues with both Ki-67 and HMG(Y) antibodies. Fig. 4, A and B, shows one of the cases of CIN2 moderate dysplasias that was immunostained in distinct experiments with the two antibodies. The lower two-thirds of the epithelium, if considered as a whole, show strong positivity in both experiments. However, a more careful inspection reveals that basal and parabasal layers are more stained by the anti-HMG(Y) antibody in comparison with Ki-67 immunostaining. This could be due to the fact that the two antibodies recognize antigens that should refer to different biological cell states (see "Discussion"). Analyses similar to those of Fig. 4, A and B, were also carried out on samples from invasive carcinomas; the results are shown in Fig. 4, C and D, and it is possible to note that immunostaining of cervix carcinomas with HMG(Y) antibody is very similar to that obtained using the Ki-67 antibody.

DISCUSSION

HMG is a family of three mammalian nuclear proteins (HMG, HMY, and HMG-C) involved in vivo in the positive or negative regulation of gene transcription (38), and a close correlation between the increase of their expression and neoplastic transformation has been reported (3–5, 26–31, 39, 40). A more direct relationship between HMG expression and malignant phenotype has been demonstrated by an antisense methodology: blocking the expression of HMG-C protein prevents the transformation of rat thyroid cell lines by viral oncogenes such as v-mos and v-ras-Ki (41). The high constitutive levels of HMG proteins of neoplastic cells prompted us and other laboratories to suggest the use of HMG expression as a marker of cellular transformation (28–31). In particular, we demonstrated that high concentrations of HMG(Y) proteins could be used as markers of the malignant phenotype of human thyroid neoplasias as well as

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* Seventy to ninety % of cells are not immunostained, the remaining cells show faint immunostaining.
* About 50% of cells show no immunostaining or faint immunostaining, whereas the remaining 50% show intermediate or strong immunostaining.
* At least 80% of cells show intermediate or strong immunostaining.

Table 1 Expression of HMG(Y) proteins in squamous intraepithelial and invasive lesions of the uterine cervix
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Fig. 3. Immunohistochemical analysis of HMG(Y) protein expression in squamous intraepithelial and invasive lesions of the uterine cervix. A, normal stratified epithelium; B, CIN 1 lesion; C, CIN 3 lesion; D, invasive carcinoma. Objective magnification, ×40. Tissues were immunostained with streptavidin/peroxidase/DAB and counterstained with hematoxylin. No immunostaining or faint immunostaining was observed in cells of normal epithelium (A). Cells of the lower third of the CIN 1 lesion (B) show intermediate or strong positivity. Most of the nuclei of the cells of the CIN 3 lesion (C) are immunostained, and strong positivity is observable in the nuclei of the invasive carcinoma (D).

Further extension of the study of the expression of HMG proteins in tumor tissues is reported in this paper dealing with squamous intraepithelial and invasive lesions of the uterine cervix. This research moves toward a wider use of the HMG(Y) antibodies as a diagnostic tool, because the aim is to correlate the level of antigen recognition with the degree of tissue alteration. With this view, we took into consideration the ectocervix of adult women, which is formed by a stratified squamous epithelium composed of basal, parabasal, intermediate, and superficial cell layers. Maturative differentiation of the epithelium takes place from the basal layer toward the superficial layer, with a progressive modification of the cellular morphology. Dysplastic processes introduce more or less severe alterations in both the epithelium and cellular morphology. Grading of dysplastic cervical epithelia is based thus far on the proportion of the epithelial thickness formed by dedifferentiated basal layer-like cells or single-cell criteria such as nuclear atypia. In our opinion, the recognition of nuclear antigens such as HMG proteins, whose expression is related to neoplastic transformation, could supply molecular information to be considered in clinical diagnoses together with the morphological observation. To this aim, we selected an antibody able to recognize the two nuclear HMG(Y) proteins on the basis of preliminary analyses carried out on cultured carcinoma cells of the uterine cervix that, according to both Northern and Western blot experiments, express the HMG(Y) gene. The results reported here show that in the analyzed samples, the level of antigen recognition parallels the CIN grade. In fact, no positivity has been detected in normal cells from smears, whereas only low levels of positivity have been found in samples from formalin-fixed paraffin-embedded normal epithelia. A progressive increase of positivity matches the progression of the epithelium lesion up to the immunohistochemical detection of all cells of the invasive carcinoma.

In this study, we also report that the antibody anti-HMG(Y) gives immunohistochemical data similar to those obtained using the antibody Ki-67. Rationalizing this result is not straightforward, because the two antibodies recognize different antigens, even if both are nuclear. Ki-67 is a nuclear antigen formed by two large polypeptides (345 and 395 kDa) that are expressed in proliferating cells but absent in resting cells (33). The function of these polypeptides, both of which are recognized by Ki-67 antibody, is still unknown, but the relationship between their expression and cell proliferation is well established (24, 33–36, 43–47). Antigen recognition by Ki-67 of tissues from the uterine cervix increases with increasing lesion grade (34, 35); our data of Table 1 and Fig. 3 show similar behavior for HMG(Y) antibody. HMG(Y) proteins are low molecular weight (about 11 kDa) nuclear proteins that are involved in both gene expression regulation and cancer (38). High levels of HMG(Y) proteins are constitutive of tumor cells and independent of cellular growth rates (3, 4, 26). Moreover, high levels of HMG(Y) proteins have been detected in both rat embryos and thyroid fibroblasts (2, 4), and more recently,
the same results have been reported in a detailed study on the embryonic development of the mouse (48). From overall consideration of the above data, we infer that the expression of the two classes of proteins [Ki-67 polypeptides and HMG(I(Y) proteins] should refer to different cellular events that, however, could be connected. Overlapping of the expression of both antigens could be present in tumor-proliferating cells or undifferentiated/dedifferentiated proliferating cells. On the other hand, it is possible that phenotype-altered cells recognized at high positivity by the anti-HMG(I(Y) antibody are detected at a low level by the Ki-67 antibody because they are not actively proliferating. This could be the case of the basal layers shown in Fig. 4, A and B. Having this scheme in mind, the cells of the cervix epithelium that recognition by Ki-67 indicates as proliferating should be formed by an altered phenotype if they are also recognized by the anti-HMG(I(Y) antibody. In conclusion, we think that immunohistochemical analyses of tissues carried out using both the HMG(I(Y) and Ki-67 antibodies can increase our understanding of the biological alterations that are present in dysplastic epithelia.

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