Correlation of c-myc Expression with Nuclear Pleomorphism in Human Renal Cell Carcinoma

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ABSTRACT

The expression of the c-myc gene product in renal cell carcinomas was examined by immunostaining with monoclonal antibody (mAb) MYC-1. The effects of preservation and fixation of tissues on staining were first examined. In cryostat sections fixed with 4% buffered formalin for 15 min, staining was observed in the nucleus. On the other hand, in paraffin sections after fixation with 10% formalin, staining was observed in the cytoplasm, but not in the nucleus. Because c-myc protein has been shown to be a nuclear protein, the finding that c-myc protein was not detectable in the nucleus appeared to be due to the preservation or fixation procedures used. Therefore, cryostat sections fixed with 4% formalin were used to investigate the correlation between the reaction of MYC-1 mAb and nuclear pleomorphism in primary and metastatic renal cell carcinomas. Among 41 primary tumors, positive staining was observed in 2 of 17 tumors (12%) of grade 1, 17 of 21 (81%) of grade 2, and all 3 (100%) of grade 3. Among 17 metastatic tumors, positive staining was not observed in any of the 5 (0%) of grade 1 but was observed in 2 of 4 (50%) of grade 2 and all 8 (100%) of grade 3. Thus, the frequency of the positive reaction with MYC-1 mAb was correlated with nuclear pleomorphism in primary and metastatic renal cell carcinomas. The reaction of Ki-67 mAb, which recognized a nuclear antigen present in proliferating cells, was also correlated with nuclear pleomorphism. These findings suggest that the c-myc gene product plays a role in cell proliferation in renal cell carcinomas.

INTRODUCTION

The c-myc oncogene was identified as a homologue of the transforming sequence of the avian myelocytomatosis virus MC29 (1). Amplification or rearrangement of the c-myc gene or promoter insertion resulting in quantitative or qualitative alterations of the gene product have been suggested as possible mechanisms of neoplastic transformation (2–5). On the other hand, the c-myc gene appears to have been highly conserved during evolution (6) and c-myc mRNA is expressed in a variety of normal and malignant cells (7).

For study of the functional role of the c-myc gene, detection of the c-myc protein with mAb2 at the cellular level is extremely useful. Evan et al. (8) prepared 6 mAbs from mice immunized with synthetic peptides, and using one of these mAbs, 6E10, Stewart et al. (9) and Sikora et al. (10) detected c-myc protein in colon and testicular cancers. In their study, the reaction of 6E10 mAb was observed in the cytoplasm, but not in the nucleus of cancer cells, although the c-myc protein appears to be a DNA binding protein (11–14). In this study, we demonstrate that, in appropriate conditions, the c-myc gene product was detected in the nucleus by immunohistological staining with MYC-1 mAb and that expression of the c-myc protein was associated with the grade of nuclear pleomorphism in primary and metastatic renal cell carcinomas.

MATERIALS AND METHODS

Tissues. Primary and metastatic renal cell carcinomas and normal kidneys were obtained from surgical or autopsy specimens in the Center for Adult Diseases, Osaka, between October 1985 and December 1987. The tissues were embedded in OCT compound (Miles Scientific, Naperville, IL) and promptly frozen on acetone and dry ice. The frozen tissues were stored at −80°C.

Monoclonal Antibodies. mAb MYC-1 was prepared from mice immunized with a truncated c-myc protein, Mr 23,000 protein, produced in Escherichia coli transfected with pTR myc 10 gene.4 The specificity of MYC-1 mAb has been reported. Briefly, in the enzyme linked immunosorbent assay, MYC-1 mAb reacted with Mr 23,000 protein, a product of the truncated c-myc gene, but not Mr 21,000 protein, a product of the ras gene, the 5' end of which was a constituent of the pTR myc 10 gene. In Western blotting, MYC-1 mAb reacted with Mr 58,000 and Mr 60,000 molecules of nuclear extract derived from HL-60 and Colo 320 cell lines, consistent with the results of others (8, 12, 15). mAb Ki-67 was prepared from mice immunized with the crude nuclear fraction of L428 cells derived from a patient with Hodgkin's disease (16) and purchased from Dako Co. (Uppsala, Sweden). It recognized a nuclear antigen present in proliferating cells, but not in resting cells. mAb K2.7 was prepared from mice immunized with the renal cell carcinoma cell line OS-RC-2 (17) and recognized the antigen localized in the cytoplasm and on the cell membrane of malignant and normal kidney cells (18). RASK-3 mAb, prepared as described elsewhere (19), reacted with the ras gene product.

Immunohistochemical Staining. Assays were performed by a modified indirect immunoperoxidase method with a Vectastain avidin-biotin complex kit for mouse IgG (Vector Laboratories, Inc., Burlingame, CA), with the following modifications. Cryopreserved tissue was sectioned on a cryostat and fixed in 4% Lillie's buffered formalin for 15 min at room temperature. Slides were blocked with 0.5% normal horse serum for 30 min and then incubated with mAbs for 60 min at room temperature. They were then washed extensively with phosphate buffered saline, and endogenous peroxidase activity was blocked by treatment with 0.6% H2O2 in methanol for 30 min. Acsites from mice bearing NS-1 myeloma cells or normal mouse serum was routinely used as a negative control.

Criteria of Nuclear Grading of Renal Cell Carcinoma. Nuclear grades were determined by the general code for clinical and pathological studies on renal cell carcinoma of the Japanese Urological Association (20): grade 1 (G1), nuclei indistinguishable from those of normal tubular cells; grade 2 (G2), moderately enlarged, often irregular and slightly pleomorphic nuclei with definite nucleoli without bizarre forms; grade 3 (G3), numerous bizarre or giant nuclei.

RESULTS

Effects of Preservation and Fixation of Tissues on the Intracellular Distribution of the c-myc Gene Product. For immuno-

Received 6/14/88; revised 2/14/89; accepted 3/22/89.

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1 This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

2 To whom requests for reprints should be addressed.

3 The abbreviation used is: mAb, monoclonal antibody.

histochemical detection of the c-myc gene product by the modified avidin-biotin complex method with MYC-1 mAb, we first studied suitable conditions for preservation and fixation of tissues to determine its actual intracellular localization. In paraffin sections of tissue fixed in 10% formalin, which are routinely used for histopathological examinations, a positive reaction with MYC-1 mAb was observed in the cytoplasm, but not in the nucleus of renal cancer cells. On the other hand, in cryostat sections fixed in 4% buffered formalin for 15 min, the positive reaction with MYC-1 mAb was observed in the nucleus (Fig. 1). Because the c-myc gene product is known to be a nuclear protein, the latter condition was used in subsequent analyses. The staining pattern and the cytoplasmic localization of the reaction with RASK-3 mAb, which detected the ras gene product used as a control, were not significantly different after these two treatments (Fig. 1).

Correlation of the Reactivity of MYC-1 mAb with the Nucleus and the Grade of Nuclear Pleomorphism in Primary and Metastatic Renal Cell Carcinomas. We examined whether the reactivity of MYC-1 mAb with the nucleus was correlated with nuclear pleomorphism in renal cell carcinomas. The nuclear pleomorphism was classified as G1, G2, and G3 according to the general code of the Japanese Urological Association (see "Materials and Methods"). The reaction of Ki-67 mAb (16), which has been shown to recognize intranuclear antigen, and that of K2.7 mAb (18), which recognizes kidney specific antigen, were also studied. In all, 41 specimens of primary renal cell carcinomas from 34 patients (including 14 specimens of different grades taken from 2 different sites of the same tumors in 7 patients) were tested for reactivities with MYC-1 mAb and K2.7 mAb. Among 41 specimens, 23 specimens from 23 patients obtained in the later period of this study were examined with Ki-67 mAb, because this antibody became available only later in the study. No deviation of the distribution of specimens of each nuclear grade was observed between these 23 specimens and the total 41 specimens. As shown in Table 1, 22 of the specimens (54%) stained positively with MYC-1 mAb, 7 (30%) with K2.7 mAb, and 39 (95%) with K2.7 mAb. Positive staining with MYC-1 mAb was observed in 2 of 17 specimens (12%) of grade 1, 17 of 21 specimens (81%) of grade 2, and all 3 specimens (100%) of grade 3. Positive staining with Ki-67 mAb was not observed in 11 specimens of grade 1 but was observed in 5 of 10 specimens (50%) of grade 2 and both specimens of grade 3. The reactions of these mAbs were clear-cut: when the reaction was positive, more than 30% of the cells in microscopic fields were stained; whereas when the reaction was negative, scarcely any cells were stained. In specimens of grade 3, both the nucleus and cytoplasm stained with either MYC-1 mAb or Ki-67 mAb. Cells of normal tubular origin and connective tissue did not stain with these mAbs. Thus, the frequency of positive reactions with MYC-1 mAb as well as with Ki-67 mAb correlated well with nuclear pleomorphism. However, no correlation was observed between the reaction with K2.7 mAb and nuclear pleomorphism. Typical stainings of specimens of the various nuclear grades with MYC-1 mAb, Ki-67 mAb, and K2.7 mAb are shown in Fig. 2.

The reactions of MYC-1 mAb, Ki-67 mAb, and K2.7 mAb with 17 metastatic renal cell carcinomas from 6 patients were also tested. As shown in Table 2, positive reactions with MYC-1 mAb, Ki-67 mAb, and K2.7 mAb were observed in 10 (59%), 7 (41%), and 16 (94%) specimens, respectively. The frequencies of positive reactions with MYC-1 mAb and Ki-67 mAb were correlated with the nuclear grades as in primary renal cell carcinomas. Moreover, the frequencies of positive reactions with these mAbs in the various nuclear grades were not significantly different in primary and metastatic renal cell carcinomas. In 3 of 6 patients (1 each of G1, G2, and G3), both primary and metastatic tissues were examined. No difference was observed in the intensities of the reactions with MYC-1 mAb of the primary and metastatic tissues of the three nuclear grades (data not shown).

DISCUSSION

Transcription of the c-myc gene was initiated in T-lymphocytes immediately after the addition of mitogen (21) and DNA synthesis or blast transformation was inhibited by addition of anti-c-myc protein antibody (22) or complementary oligonucleotide of the c-myc gene (15). Immunostaining of cultured cells suggested the nuclear localization of the c-myc protein (11, 12). Thus, the c-myc gene product has been considered to be a DNA binding protein that is expressed in association with cell proliferation. In this study, we examined tissues by immunostaining and demonstrated that the detection of the c-myc gene product in the nucleus depended on the methods of preservation and fixation of the tissue. In cryostat sections of tissues embedded in OCT compound after fixation with 4% formalin for 15 min at room temperature, the c-myc protein was detected in the nucleus. On the other hand, in paraffin sections of material fixed in 10% formalin, which are routinely used for histopathological examination, the c-myc protein was not detected in the nucleus but was detected in the cytoplasm. In the latter type of preparation, several investigators have detected the c-myc gene product in the cytoplasm by immunohistochemical studies with mouse mAb 6E10, produced by immunization with c-myc proteins (9, 10) and also with rabbit antiserum (R5452) produced by immunization with a synthetic peptide corresponding to residues 423–437 of the predicted human c-myc gene product (23). The absence of detectable c-myc gene product in the nucleus in paraffin sections thus appears to be due to an inappropriate method of preservation and fixation of tissues that results in the transfer of nuclear c-myc protein to the cytoplasm. Abnormal intracellular distributions of other proteins caused by fixation procedures have been reported (24). Another, but less likely, explanation for the absence of reaction products in the nucleus and their presence in the cytoplasm is that these mAbs, including MYC-1 mAb, detected some cross-reactive substance other than the c-myc gene product.

The nuclear grade has been used as an indicator of malignancy and is correlated with the prognosis of patients with renal cell carcinoma. We found that expression of the c-myc gene product in the nucleus was correlated with the grade of nuclear pleomorphism in primary and metastatic renal cell carcinomas. A positive reaction with Ki-67 mAb, which also recognizes a nuclear protein associated with cell proliferation, was also correlated with the grade of nuclear pleomorphism. These results suggested that renal cell carcinomas with a higher grade of nuclear pleomorphism contain more proliferative cells. The staining of the cytoplasm of specimens of grade 3 with either MYC-1 mAb or Ki-67 mAb suggested the presence of the c-myc gene product and Ki-67 antigen in the cytoplasm as well as in the nucleus in these samples. Sikora et al. (25) studied the expression of the c-myc gene in colon cancers and adjacent normal tissues and found an inverse association of its expression with histological differentiation. In a study on 15 patients, they observed greatly increased
transcription of c-myc mRNA and translation of p62 c-myc protein in 3 well differentiated tumors and the lowest transcription in 4 poorly differentiated tumors. Their results seem inconsistent with ours, but factors other than nuclear pleomorphism contributing to determination of the histological type of these tumors should be carefully considered. This point requires further study with a larger number of tumors.

A relationship between expression of the c-myc gene in the nucleus and nuclear pleomorphism in renal cell carcinoma was evident, but the genetic mechanism causing overexpression of the c-myc gene is unknown. Ocadiz et al. (26) detected amplification and/or rearrangement of the c-myc gene in most of the uterine cervical cancers they examined. However, Sikora et al. (25) did not observe amplification or rearrangement of the c-myc gene in 12 colorectal cancers, although they observed elevated levels of transcripts of the c-myc mRNA.

The degree of expression of c-myc protein in the nucleus in each grade was not significantly different in renal cell carcinomas in primary and metastatic sites. These findings suggested that expression of c-myc protein is a rather stable phenotype of neoplastic growth and is not associated with the metastatic potential. This is consistent with the finding of Wong et al. (27) that the copy number of the amplified c-myc gene and histological type of tumors in primary and metastatic sites are similar.

Table 1. Immunostaining of primary renal cell carcinomas of different grades of nuclear pleomorphism with MYC-1 mAb, Ki-67 mAb, and K2.7 mAb

<table>
<thead>
<tr>
<th>mAb</th>
<th>G1*</th>
<th>G2</th>
<th>G3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYC-1</td>
<td>2/17 (12)</td>
<td>17/21 (81%)</td>
<td>3/3 (100%)</td>
<td>22/41 (54%)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>0/11 (0)</td>
<td>5/10 (50%)</td>
<td>2/2 (100%)</td>
<td>7/23 (30%)</td>
</tr>
<tr>
<td>K2.7</td>
<td>17/17 (100%)</td>
<td>19/21 (90%)</td>
<td>3/3 (100%)</td>
<td>39/41 (95%)</td>
</tr>
</tbody>
</table>

* See "Materials and Methods" for grading (G1, G2, and G3) of nuclear pleomorphism.
* Number of specimens with positive staining/number of specimens tested.

Fig. 1. Immunostaining with MYC-1 mAb (A, C) and RASK-3 mAb (B, D) of renal cell carcinoma (grade 2) in paraffin sections after fixation in 10% formalin (A, B, × 400) and cryostat sections fixed in 4% buffered formalin for 15 min at room temperature (C, D, × 200). Staining of the nucleus with MYC-1 mAb is observed in C but not in A.

Fig. 2. Immunostaining with MYC-1 mAb (A, D, G), Ki-67 mAb (B, E, H), and K2.7 mAb (C, F, I) of renal cell carcinomas of grades 1 (A–C), 2 (D–F), and 3 (G–I) of nuclear pleomorphism. × 200.
Table 2 Immunostaining of metastatic renal cell carcinomas of different grades of nuclear pleomorphism with MYC-1 mAb, K1-67 mAb, and K2.7 mAb

<table>
<thead>
<tr>
<th>mAb</th>
<th>Positive staining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYC-1</td>
<td>G1* 0/5 (0) G2 2/4 (50) G3 8/8 (100) Total 10/17 (59)</td>
</tr>
<tr>
<td>K1-67</td>
<td>0/5 (0) 0/4 (0) 7/8 (88) 7/17 (41)</td>
</tr>
<tr>
<td>K2.7</td>
<td>5/5 (100) 3/4 (75) 8/8 (100) 16/17 (94)</td>
</tr>
</tbody>
</table>

* See Table 1, Footnote a.
+ See Table 1, Footnote b.

ACKNOWLEDGMENTS

We thank Drs. S. Ishigami and H. Akedo for help during this study.

REFERENCES


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