Determination of Ki-67 Defined Growth Fraction by Monoclonal Antibody MIB-1 in Formalin-Fixed, Paraffin-Embedded Prostatic Cancer Tissues

Marinus A. Noordzij, Theodorus H. van der Kwast, Gert Jan van Steenbrugge, Wytske M. van Weerden, Maria H.A. Oomen, and Fritz H. Schröder

Departments of Urology (M.A.N., G.J.v.S., M.H.A.O., F.H.S., W.M.v.W.) and Pathology (T.H.v.d.K.), Erasmus University, Rotterdam, The Netherlands

ABSTRACT: The applicability of MIB-1, a monoclonal antibody directed against the Ki-67 antigen, was studied in the PC-82 and LNCaP prostatic tumor models at various levels of proliferative activity. Statistically significant correlations were found in LNCaP cultures between Ki-67 and MIB-1 scores (r = 0.84, $P < 0.001$), and in PC-82 tumors between MIB-1 scores and paraffin tissue Ki-67 (pKi-67) (r = 0.90, $P < 0.001$), frozen tissue Ki-67 (fKi-67) (r = 0.86, $P < 0.001$), and BrdU uptake (r = 0.70, $P < 0.001$), respectively. pKi-67 scores were double the fKi-67 scores, which may be due to methodological differences. MIB-1 scores exceeded both the fKi-67 and pKi-67 scores. The affinity of MIB-1 for the antigen is much higher than the affinity of Ki-67, which may explain the differences. MIB-1 is a promising means of evaluating the presence of only minute amounts of the Ki-67 antigen in paraffin-embedded human tumor material, especially in relatively slowly growing tumors. © 1995 Wiley-Liss, Inc.

KEY WORDS: proliferative activity, PC-82, LNCaP, BrdU, immunohistochemistry

INTRODUCTION

Since its description in 1983, much attention has been paid to the mouse monoclonal antibody Ki-67 [1]. Ki-67 is directed against a nuclear antigen expressed in human cells during the G1, S, G2, and M phases of the cell cycle, but not during the G0 phase [2]. Quiescent (G0) cells which enter the cell cycle do not express the antigen in the early G1 phase [2]. The proliferative activity of a number of human tumors, including prostatic carcinoma, has been studied by the application of Ki-67, as investigated by Oomens et al. [3] and reviewed by Brown and Gatter [4]. Routine studies were hampered by the fact that Ki-67 was thought to be applicable only to fresh-frozen tissues. MIB-1 is a recently described mouse monoclonal antibody raised against recombinant parts of the Ki-67 antigen, and it is applicable to routinely processed, paraffin-embedded tissues following antigen retrieval [5,6]. It has recently been shown that Ki-67 also reacts with paraffin tissue sections, using this technique [7].

DNA-replicating cells can be identified by a monoclonal antibody to BrdU (bromodeoxy-uridine), a thymidine analogue incorporated into DNA in the S phase of the cell cycle [8]. BrdU-uptake has been studied in human tumors [9–11]. The present study was performed to investigate the applicability of MIB-1 to routinely processed prostate cancer specimens.

Two human prostatic tumor models were used in the present study: the PC-82 xenograft model which is serially transplantable in athymic nude mice [12], and the in vitro LNCaP cell line [13]. The growth rate of both hormone-dependent tumor models can be manipulated by growing the tumors at various an-

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drogen concentrations [14,15]. The potency of using the Ki-67 antibody for monitoring hormonal responses in the PC-82 tumor has been described previously [16].

In the present study, MIB-1 scores were correlated with fKi-67 (frozen tissue Ki-67), pKi-67 (paraffin tissue Ki-67), and BrdU scores. This was achieved by application of the antibodies to frozen or paraffin tissue sections from the same PC-82 tumor, or to slides with acetone-fixed LNCaP cells. Hormonal manipulation of the PC-82 tumors and LNCaP cells resulted in tissues at various levels of proliferative activity. Also, a number of radical prostatectomy specimens embedded 6–16 years ago were stained with MIB-1 in order to investigate the applicability of MIB-1 to archival clinical tissues.

MATERIALS AND METHODS

Experimental Models

PC-82 tumor cells were subcutaneously implanted in nude mice of Balb/c background. Tumor growth was manipulated by implantation of tumor-bearing mice with various androgen levels; tumor growth could be arrested by androgen withdrawal [14]. BrdU (10 mg/kg) was injected intraperitoneally 1 hr prior to sacrifice of the host animal. After sacrifice of the animal, tumor sections were either snap-frozen in liquid nitrogen-chilled isopentanyl 99% and stored at −80°C, or routinely fixed in 4% formalin in PBS (phosphate-buffered saline, pH 7.4) and embedded in paraffin. Routinely processed prostatectomy specimens embedded 6–16 years ago were obtained from the Department of Pathology, Dijkzigt Hospital, Rotterdam. To avoid the risk of detachment of cells or tissues from the glass slides, APES (3-aminopropyl-triethoxysilane)-coated slides were used in all experiments [17].

LNCaP-FGC (fast-growing colony) cells were seeded on APES-coated slides. The cells were grown in 7.5% fetal calf serum at standard conditions as described elsewhere [18]. The culture medium from exponentially growing cultures was replaced (after 2 days) by 5% DCC (dextran-coated charcoal)-treated, i.e., androgen-depleted, serum. Slides were fixed in acetone for 10 min at 0, 24, 48, 72, and 96 hr following androgen withdrawal. The LNCaP experiments were performed in triplicate.

Frozen Tissue Ki-67 (fKi-67) and BrdU Staining

Ki-67 and BrdU staining procedures have been described previously [3,8]. Briefly, monoclonal antibody Ki-67 (DAKO, Glostrup, Denmark) and a monoclonal antibody specific for BrdU (Eurodiagnostics,

Apeldoorn, The Netherlands) were applied to 5-μm thick frozen and paraffin sections, respectively. Ki-67 was diluted 1:5 in PBS. Antibody-antigen binding was visualized using an indirect two-step peroxidase method with DAB (3,3'-diaminobenzidine tetrahydrochloride) as chromogen. Prior to application of the BrdU antibody, these sections were subsequently placed in 2 N HCl for 30 min to uncoil the DNA, and in a borate buffer, pH 8.5, for neutralization [8].

Paraffin Tissue Ki-67 (pKi-67) and MIB-1 Staining

The procedure used for MIB-1 staining is the result of the optimization of the procedure recommended by the manufacturer (Immunotech, Marseille, France). An identical procedure was used for pKi-67 staining, except for the primary antibody. Paraffin PC-82 and prostatectomy sections of 5-μm thickness were cut and mounted on APES-coated slides [17]. The slides were kept overnight at 60°C. After rehydration, the slides were placed in 10 mM citrate buffer adjusted to pH 6.0, after which antigen retrieval was performed in a microwave oven at 700 W for 15 min [6]. Distilled water was regularly added to prevent drying of the slides due to evaporation. The slides were allowed to cool down to room temperature, and were subsequently rinsed in PBS. Antigen retrieval was omitted for the acetone-fixed LNCaP cells. To avoid nonspecific staining, both tissue sections and LNCaP cultures were preincubated for 15 min at 37°c with normal goat serum (DAKO) diluted 1:10 in PBS. Overnight incubation with MIB-1 (Immunotech), diluted 1:200 in PBS, or with Ki-67 (DAKO), diluted 1:10 in PBS, was carried out at 4°C. After rinsing in PBS, the sections were incubated for 30 min at room temperature with a 1:400 dilution of biotinylated goat anti-mouse serum (DAKO) in PBS containing 3% normal goat and 3% normal human serum. After rinsing in PBS, a final incubation with avidin biotin complex (ABC), consisting of streptavidin and biotinylated horseradish peroxidase diluted in PBS, as described by the manufacturer (DAKO), was performed for 30 min at room temperature. The staining was visualized with 0.075% DAB (Fluka, Buchs, Germany) in PBS with 0.08% hydrogen peroxide as substrate for 7 min. Sections were rinsed in distilled water, and counterstained with Mayers hematoxylin. After dehydration, sections were mounted with malinol.

Quantification

Sections were examined at 400× magnification. The numbers of positive and negative tumor cell nuclei in the 4 × 4 central part of a 10 × 10 grid inserted in one of the oculars were counted. Adjacent microscopical fields were examined until 1,000 or more
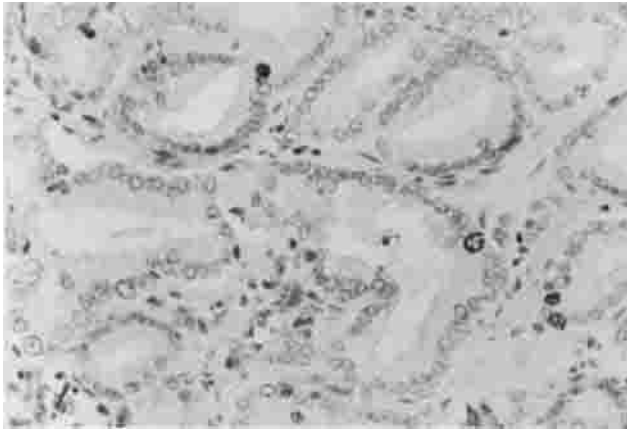
Fig. I. Well-differentiated adenocarcinoma in a tissue section of a 16-year-old radical prostatectomy stained with MIB-1. ×270.

cells were counted. Cells with an apparent brown staining of the nucleus and well-demarcated nucleoli were judged positive. Scores are presented as percentages of cells counted. Spearman's rank correlations were calculated since the data were not normally distributed. For statistical confirmation the Student's t-test was performed using the software package SPSS 4.01 (Statistical Package for Social Sciences, SPSS, Inc., Chicago, IL).

RESULTS

Immunostaining of Prostatectomy Specimens

Figure 1 shows MIB-1 staining in a 16-year-old prostatectomy specimen. Although variations in staining intensity were observed, positive and negative cells were generally easy to identify. Occasionally cytoplasmic staining was observed. Detachment of parts of the prostatectomy sections from the glass slide was seen, especially in sections containing the (fatty) periprostatic tissue. The risk of detachment decreased when the slides, prior to antigen retrieval, were kept overnight at 60°C. This procedure did not alter the immunoreactivity for MIB-1.

Immunostaining of Prostatic Cancer Cell Lines

Hormonal manipulation of the PC-82 tumor-bearing mice resulted in tumors with various growth rates, which were clearly reflected by the MIB-1-, frozen Ki-67 (fKi-67)-, paraffin Ki-67 (pKi-67)-, and BrdU-defined proliferative activities (data not shown). Intensity of MIB-1 staining was in general more pronounced than intensity of fKi-67 and pKi-67 staining. Weak nuclear MIB-1 staining could still be observed when the antibody was diluted 1:5,000 (0.04 μg/ml). Ki-67 staining decreased at a concentration of 3.96 μg/ml (1:50 dilution). Only little

intratumoral variation in the dispersion of positive nuclei was observed. MIB-1 scores ranged from 0.1–25.7% (mean 11.6%), fKi-67 scores ranged from 0.2–6.8% (mean 2.9%), pKi-67 scores ranged from 0.6–18.5% (mean 6.7%), and BrdU scores ranged from 0.0–7.5% (mean 2.4%). Figure 2 shows fKi-67 staining (Fig. 2A), pKi-67 staining (Fig. 2B), and MIB-1 staining (Fig. 2C) in sections taken from one PC-82 tumor. In PC-82 tissues a highly significant correlation was found between MIB-1 and fKi-67 scores (r = 0.86, $P < 0.001$, n = 21), and between MIB-1 and pKi-67 scores (r = 0.90, $P < 0.001$, n = 25), as depicted in Figure 3. Relatively weak correlations were found between MIB-1 scores and BrdU-uptake, as shown in Figure 4 (r = 0.70, $P < 0.001$, n = 24), and between fKi-67 scores and BrdU-uptake (r = 0.44, $P = 0.052$, n = 20). The correlation between fKi-67 and pKi-67 scores was 0.77 ($P < 0.001$, n = 21). MIB-1 scores exceeded both the fKi-67 scores (linear regression coefficient β = 3.67, 95% confidence interval (CI): 2.87–4.47) and the pKi-67 scores (β = 1.57, CI: 1.28–1.86). pKi-67 scores were double the fKi-67 scores (β = 2.03, CI: 1.31–2.75).

The acetone-fixed LNCaP cultures did not require antigen retrieval prior to application of MIB-1. The growth-inhibiting effect of androgen depletion on the LNCaP cells was clearly reflected by a loglinear decline of Ki-67 and MIB-1 scores, representing the exit of cells from the cell cycle (Fig. 5). The MIB-1 and Ki-67 scores observed in LNCaP cultures were generally larger than those observed in PC-82 tissue sections, and ranged from 16–70% (mean, 39.6) for MIB-1, and from 7–41% (mean, 19.8) for Ki-67. MIB-1 scores were almost double the Ki-67 scores (β = 1.81, CI: 1.44–2.18), and the correlation coefficient was 0.84 ($P < 0.001$, n = 15).

DISCUSSION

The present study was conducted to compare the novel proliferation marker MIB-1 with frozen tissue-based Ki-67 (fKi-67), paraffin tissue-based Ki-67 (pKi-67), and BrdU uptake. This was achieved by immunostaining samples derived from hormonally manipulated PC-82 tumors and LNCaP cell cultures with the different antibodies [1,5,7,8]. In LNCaP cultures only Ki-67 and MIB-1 were studied. MIB-1 staining was also studied in archival radical prostatectomy specimens.

Antigen retrieval is essential for immunostaining with the MIB-1 or the Ki-67 (pKi-67) antibodies when applied to paraffin-embedded tissue sections. As found by others [19], application of MIB-1 without antigen retrieval only stained mitotic cells (not shown). The mechanism of antigen retrieval remains unclear as yet, but it has been suggested that the
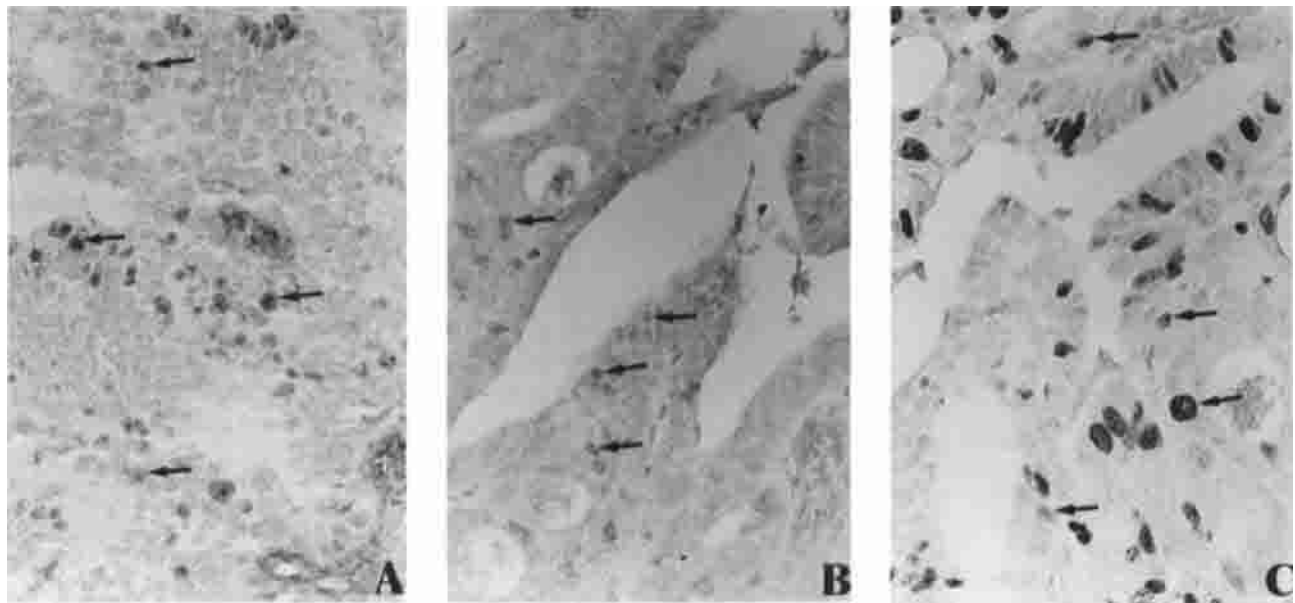
Fig. 2. Immunostaining of a PC-82 tumor sample (grown in a hormonally untreated mouse). **A:** Fresh-frozen section, Ki-67 (fKi-67). **B:** Paraffin-embedded section, Ki-67 (pKi-67). **C:** Paraffin-embedded section, MIB-1 (×270). Some positive nuclei are indicated with arrows.

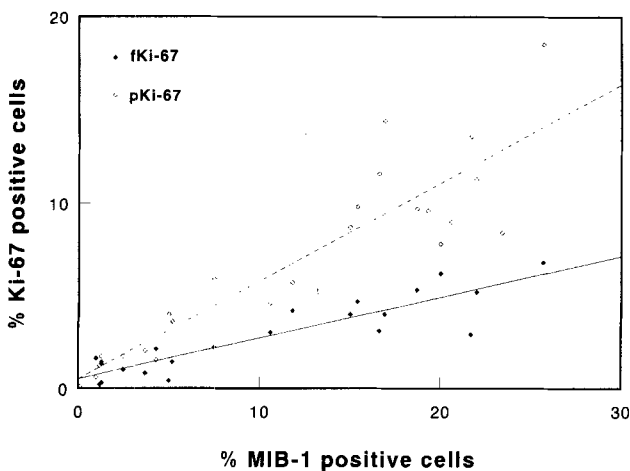% MIB-1 positive cells

% MIB-1 positive cells

Fig. 3. Correlations between MIB-1 scores and the respective fKi-67 (frozen tissue-based Ki-67) scores and pKi-67 (paraffin tissue-based Ki-67) scores, assessed on PC-82 tumor samples (n = 21). Note the difference in scaling of the axes. Regression lines: fKi-67 (solid line), pKi-67 (dotted line).
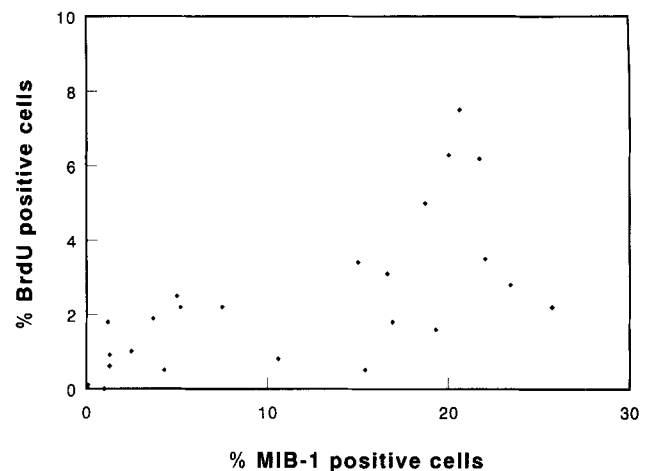
Fig. 4. Correlation between MIB-1 scores and BrdU-uptake in 24 PC-82 tumors. Note the difference in scaling of the axes.

formalin-induced protein crosslinkings are broken with this procedure, thus unmasking the epitopes of the various antigens [6]. In the present study MIB-1 staining could still be observed in a 16-year-old prostatectomy specimen. Cattoretti et al. [19] detected MIB-1 reactivity in a 60-year-old Zenkers' fixed lymph node. Cytoplasmic staining was occasionally observed in some of the prostatectomy specimens. A similar cytoplasmic staining has also been described

in prostatic tissues stained by Ki-67, but appears not to be associated with proliferation [20]. fKi-67, pKi-67, and especially MIB-1 staining were not always equal in intensity, as Figure 2 shows. This may complicate the distinction of positive and negative cells. In the present study, cells were scored positive only when both nuclear and nucleolar staining were visible. If cells showing only one of these patterns were included, scores would have been higher in a number of the sections.

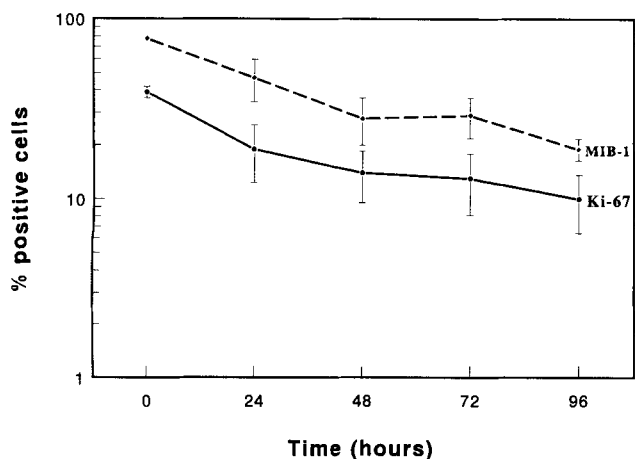Strong and statistically significant correlations were found between MIB-1 and fKi-67 scores (r =

Fig. 5. The relation of MIB-1 (dotted line) and Ki-67 scores (solid line) to time in a time-course experiment with hormone responsive LNCaP cells following androgen withdrawal at day 0 (mean ± SD).

0.86, $P < 0.001$), between MIB-1 and pKi-67 scores ($r = 0.90$, $P < 0.001$) in PC-82 tumors (Fig. 3), and between MIB-1 and Ki-67 scores ($r = 0.84$, $P < 0.001$) in LNCaP cultures (Fig. 5). Surprisingly, MIB-1 scores exceeded both the fKi-67 scores ($\beta = 3.67$) and the pKi-67 scores ($\beta = 1.57$) in PC-82 sections. The methodological differences between the fKi-67 and MIB-1 staining procedures probably partially account for this discrepancy. However, the staining procedures were identical for Ki-67 and MIB-1 applied to paraffin PC-82 sections ($\beta = 1.57$) and LNCaP cultures ($\beta = 1.81$). Still, a marked difference was observed in both cases as demonstrated by the regression coefficients. The described intensity difference between MIB-1 and Ki-67 labelling might partially account for this discrepancy. McCormick et al. [21] have also shown a close relationship between MIB-1 and Ki-67 scores. However, based upon other types of tissue, they did not describe the discrepancy found in the present study. It has very recently been shown that the nuclear Ki-67 protein, i.e., the protein recognized by Ki-67, can exist free or associated with double-strand DNA [22]. This study demonstrated that, in contrast to Ki-67, MIB-1 recognizes the free protein. Moreover, the affinity of MIB-1 for the DNA Ki-67 protein complex largely exceeds the affinity of Ki-67 for that complex [22]. This indicates that binding of the Ki-67 protein to the DNA modulates its conformation, which makes it an antigen for both Ki-67 and MIB-1. Since MIB-1 was raised against recombinant expressed Ki-67 protein, it was to be expected that MIB-1 would also recognize free Ki-67 protein. These results provide at least one plausible explanation for the differences between the Ki-67 and MIB-1 scores

found in the present study. Furthermore, weak nuclear staining could still be observed when MIB-1 was applied at a dilution of 1:5,000 (0.04 μg/ml), whereas nuclear Ki-67 staining decreased at a dilution of 1:50 (3.64 μg/ml).

Expectedly, BrdU pulse labeling scores are smaller than Ki-67 and MIB-1 scores [9,23]. Van Dierendonck et al. [24] have shown that Ki-67 scores cannot automatically be used to determine the growth fraction of a tumor. Therefore, the relatively weak correlations found between Ki-67 and BrdU, and between MIB-1 and BrdU, are not surprising. Sasaki et al. [9] described a correlation coefficient of 0.89 between Ki-67 and BrdU scores in 20 malignant human tumors. The proliferation scores in their study (1.9–37.5% for Ki-67 and 1.6–23.4% for BrdU) were generally larger than the scores found in the present study. In a study of Van Weerden et al. [23] using the PC-82 tumor model, the correlation between Ki-67 and BrdU scores was comparable to that found in the present study. The clinical use of BrdU uptake is hampered as administration of BrdU to a patient may cause acute toxic reactions and/or chromosomal abnormalities [25,26].

In conclusion, MIB-1 is a promising antibody and allows for the detection of very small amounts of the Ki-67 antigen in routinely processed, paraffin-embedded archival tissues. MIB-1 might be of special value in tumors with relatively small growth fractions, such as prostatic carcinomas. At our institution a retrospective study to determine the prognostic value of MIB-1 in prostate cancer patients is currently in progress.

ACKNOWLEDGMENTS

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