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Short Communication

A New Proliferation-Associated Nuclear Antigen Detectable in Paraffin-Embedded Tissues by the Monoclonal Antibody Ki-S1

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A monoclonal antibody (Ki-S1) has been raised that reacts with the nuclei of proliferating cells. The antigen recognized is resistant to formalin fixation and can be detected in frozen tissues as well as in routinely processed specimens. In immunohistochemistry, nuclear staining can be seen in those tissues and cellular compartments known to be actively proliferating. Peripheral blood lymphocytes are negative but show a strong increase in antigen expression after mitogen stimulation. Flow cytometric determination of DNA content and antigen expression revealed negativity of G_0 cells and positivity of G_1 to G_2/M cells. A cytoplasmic co-reactivity, not associated with proliferation, was confined to Langerhans islands of the pancreas. The nuclear localized antigen has a molecular mass of 160 kd and therefore seems to be different from all other known immunohistochemical markers of proliferating cells. We conclude that the monoclonal antibody Ki-S1 might provide a useful tool for studying cell proliferation in situ under normal and pathological circumstances. (Am J Pathol 1993, 142:3–10) (Am J Pathol 1993, 142:000–000)

The clinical assessment of cell proliferation and growth in human tissues is achieved by various methods. Among the methods in use are mitotic count,[1] thymidine labeling index[2] and bromodeoxyuridine incorporation,[3] nucleolar organizer regions,[4] and flow cytometry.[5] Whereas each of these procedures has important shortcomings with regard to reproducibility and performance under routine circumstances,[6,7] immunohistochemical methods have provided operational markers of cell proliferation. Proliferating cell nuclear antigen (PCNA) is resistant to formalin fixation and has been used in several studies as an immunohistochemical instrument for the detection of cycling cells.[8,9] The antigen recognized has been identified as the auxiliary protein of DNA polymerase delta.[10] However, there is growing evidence that in tumor cells a deregulated expression of the corresponding antigen may occur.[11,12] Therefore, it has been suggested that PCNA should only be used with caution as a marker for the tumor growth fraction.[11,12] Another cell cycle–related antigen recognized by the monoclonal antibody Ki-67[13] has proven its utility as an operational marker of cell proliferation in a number of studies.[14] The Ki-67 antigen is expressed by normal and neoplastic cells at G_0-G_1 transition and then throughout the cell cycle, with maximum expression in the M phase.[15] Immunohistochemically, the antibody selectively recognizes nuclei of proliferating cells and exhibits a cytoplasmic co-reactivity with the basal layer of stratified epithelium,[13] probably due to a cross reactivity with cytokeratins. The corresponding antigen has partly been identified.[16] In a number of studies Ki-67 has been used to correlate growth fraction with clinical outcome and a significant relationship could be demonstrated in different tumors.[14] One major drawback of the Ki-67 epitope is that it is rapidly destroyed by fixation, which has prevented its wider application as an immunohistochemical prognostic marker in tumors.[6,7,14] In this study we present a new marker for proliferating cells, desig-

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nated Ki-S1, which displays a cell cycle–related expression similar to that of Ki-67, but which, by contrast, does not bind to the cytoplasm of basal layer cells in stratified epithelium and which is resistant to formalin fixation and thus can be used in retrospective studies on archival tissue specimens.

Materials and Methods

Generation of Ki-S1 Antibodies

The monoclonal antibody Ki-S1 was generated by somatic hybridization of P_3×63-Ag8.653 mouse myeloma cells with spleen cells from BALB/c mice. Immunization, somatic hybridization, and screening for antibody secreting hybrid clones were done as described by Radzun et al.[17] using Triton-X 100 lysed U-937 cells[18] as immunogen. Eight clones producing antibodies reactive with nuclei of proliferating cells were selected and subcloned followed by retesting. One of the hybrid clones (Ki-S1) produced antibodies which immunostained nuclei in paraffin-embedded tissues. Reactivity of the antibody was tested on paraffin-embedded samples from all human tissue types.

Determination of Molecular Mass

Radiolabeling and immunoprecipitation of L428 cells[19] or mitogen-stimulated peripheral lymphocytes were done as recently described by Hansmann et al[20] with minor modifications. After brief lysation of the cells with 2% Triton X-100, the nuclei were separated by centrifugation (12,000*g*) and two further washings followed by disintegration of the nuclei with 0.35 M NaCl (30 min, 4 C). The obtained supernatant as well as the cytoplasmic lysate were used for immunoprecipitation with Ki-S1 and anti-mouse Ig coupled to Protein A-sepharose (Biochrom, Berlin, Germany). The immunoprecipitate was further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of the gels. A molecular weight standard (Sigma, Munich, FRG) served as reference.

In addition to immunoprecipitation, Western blot experiments were performed to determine the molecular mass. The L540 cell-line[19] was used in addition to the above-mentioned cells. Cells were lysed by snap-freezing in liquid nitrogen, thawing, and boiling in an SDS-sample buffer. Proteins were then electrophoretically separated in polyacrylamide gels (7.5–15%). The Western blot transfer to nitrocellulose membranes lasted 16 hours. After being blocked with 3% bovine serum albumin, the membranes were incubated with Ki-S1 antibody (undiluted supernatant) for 2 hours. Visualization was done with 4-chloro 1-naphtol after a 2-hour incubation period with peroxidase-conjugated rabbit anti-mouse IgG.

Flow Cytometric Analysis

Normal human peripheral blood lymphocytes were separated as described and stimulated with phytohemagglutinin A for 24, 48, and 72 hours.[21] After harvesting, the cells were fixed for 1 hour with 4% (vol/vol) paraformaldehyde at 4 C. Cells were permeabilized by 0.1% (vol/vol) Triton-X 100 in phosphate-buffered saline (PBS). Subsequently, cells were incubated with Ki-S1 for 60 minutes followed by anti-mouse $F(ab')_2$-FITC labeling (Boehringer, Mannheim, Germany).[22] Analysis was performed on an Ortho 50H cell sorter connected to a 2151 computer system as described previously.[22] The antibody-specific fluorescence was registered on a log scale encompassing 3.5 decades. In some experiments Ki-67 monoclonal antibody[13] was used as control.

Immunohistochemistry

Immunohistochemical stainings were performed as described with minor modifications.[17] Briefly, 2 to 4 µ sections were deparaffinized in xylol, rehydrated, and incubated for 5 minutes with 3% hydrogen peroxide in methanol, followed by treatment with 0.1% trypsin (Sigma, Steinheim, Germany) for 45 minutes. After rinsing, Ki-S1 antibody concentrated to 15 µg/ml and diluted 1:60,000 to 1:100,000 was added and incubated for 30 minutes. Visualization was performed with the avidin biotin complex using peroxidase and rabbit anti-mouse antibody (all reagents were purchased from DAKO, Hamburg, Germany).

Results

Proliferation-Associated Expression of the Ki-S1 Antigen in Normal Tissues

Ki-S1 staining was almost entirely restricted to the nucleus; only a weak cytoplasmic staining could occasionally be observed. The distribution of nuclear Ki-S1 immunoreactivity in non-neoplastic tissues was consistent with the corresponding antigen being associated with cell proliferation. Staining was seen in those tissues and cellular compartments known to be actively proliferating. For example, Ki-S1 antigen expression was displayed by germinal centers of lymphoid tissues; the antigen was present in the nuclei of the basal layer of stratified epithelium, and

in the crypts of intestinal epithelium. In the testis, the majority of spermatogonia were stained, but spermatids and sperm were unstained, as were interstitial cells and Sertoli cells. In the bone marrow, the nuclei of immature precursor cells of erythropoiesis and granulopoiesis exhibited intensive staining. By contrast, the segmented nuclei of mature granulocytes revealed no immunoreactivity (Figure 1). A single major cytoplasmic co-reactivity not associated with proliferation occurred in the Langerhans islands of the pancreas. A weak nuclear background staining not associated with proliferation was occasionally seen in chondrocytes of hyaline cartilage.

The antigen proved to be resistant to a number of tissue fixatives including ethanol, methanol, and formalin. In order to evaluate the sensitivity of the antigen versus prolonged formalin fixation, human tonsils were subjected to fixation in unbuffered formalin (10% vol/vol) for 6 to 72 hours. No significant differences in staining intensity could be observed between the different fixation periods within this time interval. Staining of cryostat sections and formalin-fixed tissue rendered almost identical results with a slight reduction of staining intensity in formalin-fixed tissue specimens. Methanol/acetic acid fixation led to a destruction of the antigen.

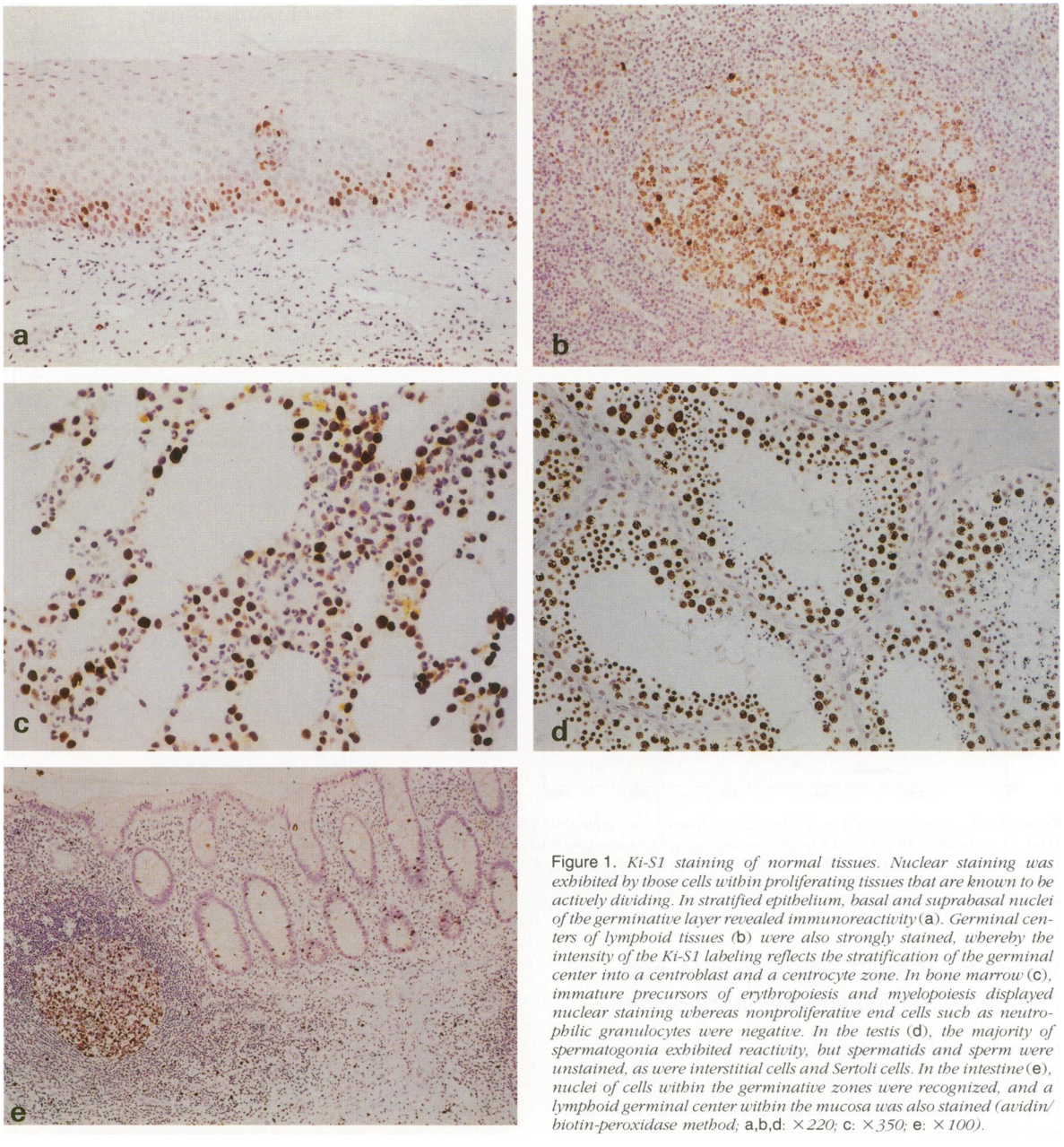

Figure 1. *Ki-S1 staining of normal tissues. Nuclear staining was exhibited by those cells within proliferating tissues that are known to be actively dividing. In stratified epithelium, basal and suprabasal nuclei of the germinative layer revealed immunoreactivity* (a). *Germinal centers of lymphoid tissues* (b) *were also strongly stained, whereby the intensity of the Ki-S1 labeling reflects the stratification of the germinal center into a centroblast and a centrocyte zone. In bone marrow* (c), *immature precursors of erythropoiesis and myelopoiesis displayed nuclear staining whereas nonproliferative end cells such as neutrophilic granulocytes were negative. In the testis* (d), *the majority of spermatogonia exhibited reactivity, but spermatids and sperm were unstained, as were interstitial cells and Sertoli cells. In the intestine* (e), *nuclei of cells within the germinative zones were recognized, and a lymphoid germinal center within the mucosa was also stained (avidin/biotin-peroxidase method;* a,b,d: ×220; c: ×350; e: ×100).

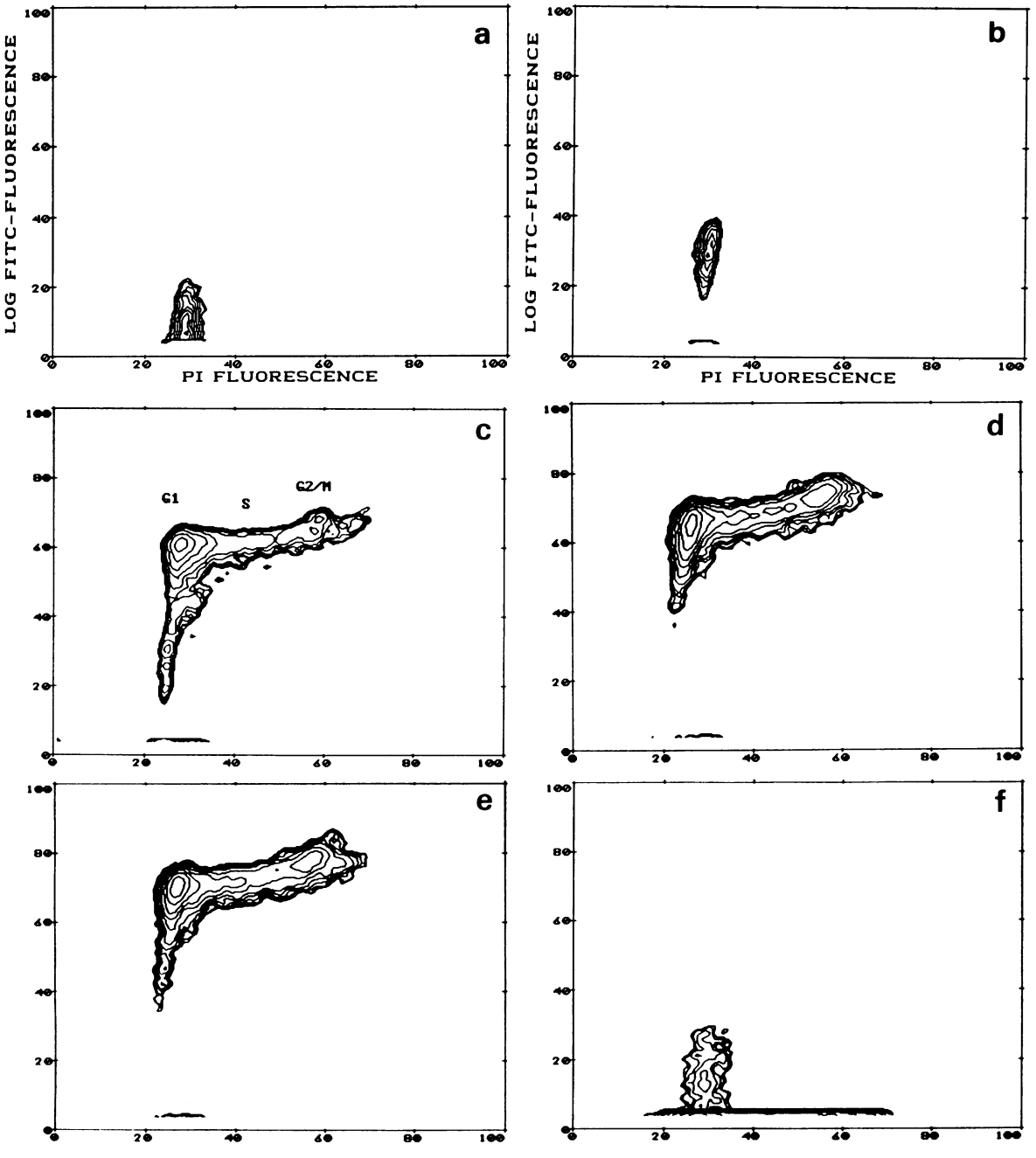

Figure 2. *Flow cytometry of mitogen-stimulated lymphocytes after DNA staining and Ki-S1 immunostaining. Freshly isolated lymphocytes did not show Ki-S1 binding* (a). *After 24 hours of stimulation* (b) *increased background staining can be observed , yielding similar results to the control without primary antibody* (f). *After 48 hours most of the lymphocytes are Ki-S1 positive* (c). *G_1, S, and G_2/M cells exhibit antigen expression* (c). *When the stimulation was proceeded for 72 hours almost all the cells had entered the cell cycle and became Ki-S1 positive* (d). *No significant differences could be observed when the same 72-hour-stimulated lymphocytes were stained with Ki-67* (e). *Channel numbers 0–100 are given on the ordinate axis, approximately 30 channels are corresponding to one log decade, 100 channels are corresponding to 3.5 log decades.*

Induction of Ki-S1 Expression During Mitogenic Stimulation of Lymphocytes

Normal peripheral lymphocytes, which are known to represent nonproliferative G_0 cells, exhibited no Ki-S1 positivity. Phytohemagglutinin-induced blastic transformation, however, was accompanied by increasing Ki-S1 expression 24 to 48 hours after stimulation. Flow cytometric analysis of cell cycle–related antigen expression showed G_1-G_2/M cells to be positive, with maximum expression in the G_2 and M-phase (Figure 2c–d). No significant antibody binding could be detected in freshly isolated and 24-hour-stimulated lymphocytes, the latter showing only a slight increase of fluorescence intensity over the background. After 48 hours, more than 50% of

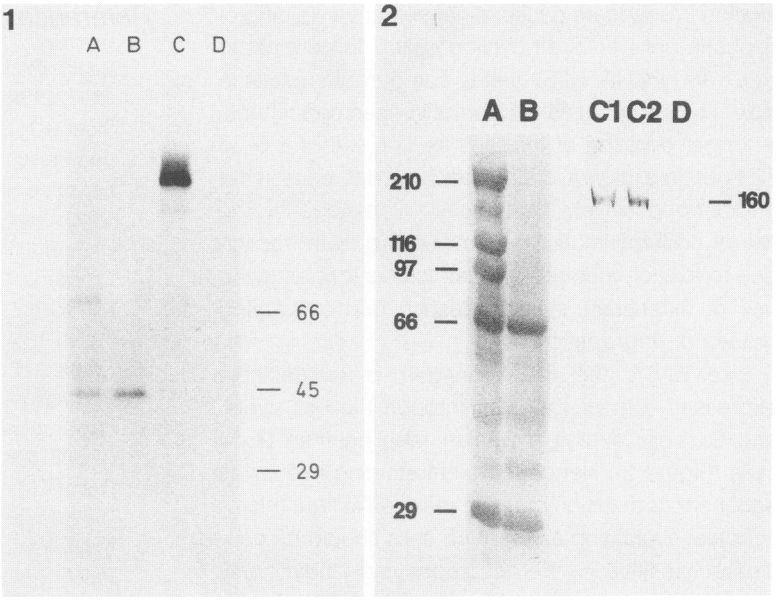

Figure 3. *Determination of the molecular mass of the Ki-S1 antigen: immunoprecipitation* (**1**) *of separated L428 nuclei yielded a 160 kd protein and a minor variant of approximately 140 kd* (**c**). *In the cytoplasm a protein of approximately 68 kd could be immunoprecipitated* (**a**). *Controls without primary antibody showed a 45-kd unspecific protein in the cytoplasm* (**b**) *and no unspecific reaction in the nuclear fraction* (**d**). *Western blot* (**2**) *analysis of L428 and L540 cells revealed a 160 kd protein* (**c1, c2**) *to that Ki-S1 bound. A control without primary antibody is given in* **d** *and molecular weight standards are shown in* **a** *and* **b**.

cells were positive. The proportion of Ki-S1 positive lymphocytes and the quantitative nuclear Ki-S1 expression increased further, and in 72-hour-cultivated lymphocytes more than 80% of the cells exhibited nuclear staining (Figure 2d).

Determination of the Molecular Mass of the Ki-S1 Antigen

The molecular mass of the Ki-S1 antigen was determined in normal peripheral lymphocytes and two tumor cell lines. No differences between normal lymphocytes and cell lines could be observed, and immunoprecipitation and Western blot analysis yielded similar results. Immunoprecipitation of purified nuclei from labeled cells rendered a major 160 kd protein. In addition, a minor variant of 140 kd was co-immunoprecipitated (Figure 3, panel 1c). In the immunoprecipitation of the cytoplasmic fraction, a single minor protein of 68 kd could constantly be observed (Figure 3, panel 1a). By contrast, Western blot analysis of whole lysed cells yielded a single 160 kd protein in stimulated lymphocytes as well as in the cell lines used. The minor protein variants observable in the immunoprecipitates of the nuclear and cytoplasmic cell fraction could not be detected (Figure 3, panel 2c).

Discussion

Proliferation is considered to be an important feature characterizing the malignant phenotype and biological behavior of tumors.[6,7] Hence, attempts have been made to establish parameters of tumor cell growth. Due to specific shortcomings none of the methods developed so far, such as mitotic figure or nuclear organizer region counts, thymidine or bromodeoxyuridine incorporation, or DNA flow cytometry, has encountered general acceptance as an instrument of routine surgical pathology.[6,7]

Immunohistochemical methods to determine proliferation offer the advantage of negligible tissue consumption and of *in situ* analysis which allows differentiation of proliferating tumor and stromal cells.[6,7,14,23,24] The Ki-67 antigen and PCNA have been used as immunohistochemical markers of proliferating cells. The Ki-S1 antigen is 160 kd in size (Figure 3) and therefore seems to be clearly different from these known cell cycle–associated antigens. The molecular mass of the Ki-67 antigen is 345 kd and 395 kd[16] whereas PCNA is considerably smaller (36 kd).[10] The distribution of the Ki-S1 antigen in normal tissues reveals that it is almost entirely restricted to the nuclei of proliferating cells (Figure 1). Consequently the Ki-S1 antigen represents a novel immunohistochemical marker for proliferating cells.

However, a number of caveats have to be kept in mind when immunohistochemical determination of growth fraction is considered. First of all, the proliferation-associated antigen under investigation might undergo degradation or chemical denaturation caused by fixation. Since the amount of Ki-S1 antigen increases from G_1 to M phase and is negative in G_0 phase (Figure 2), the loss of antigen due to degradation might lead to a pseudonegativity of early G_1 cells with a low amount of antigen. Hence, varying grades of tissue preservation will create different thresholds of detection and consequently cause inconstant proliferation indices. With PCNA the situation is even more complicated because alternative

fixation procedures cause either selective staining of S phase cells or of all cycling cells.[8] In contrast to Ki-67 and PCNA, Ki-S1 yields comparable results in frozen tissue sections and formalin-fixed specimens. A longer duration of formalin fixation within a 6- to 72-hour time interval did not lead to a decrease in the number of positively stained cells. Consequently, the risk of underestimation of tumor cell growth fraction due to fixation-induced antigen loss is low, as is the risk of inconstant staining results due to varying grades of degradation.

Like Ki-67, the Ki-S1 antigen is increasingly expressed with progression through the cell cycle, and G_1 cells exhibit a weaker labeling than G_2/M cells (Figure 2). Hence, different staining intensities occur and a decision must be reached in light microscopical evaluation as to which cells should be considered positive. In the accompanying study[25] only intensely labeled cells were counted, yielding a significant correlation with the S phase fraction.

Furthermore, the immunohistochemical approach to assessing proliferation cannot determine cell cycle length. The proliferation rate of a cell population may depend on this parameter as well as on the growth fraction. Ki-S1 can provide information only on the latter. A number of studies have pointed to tumor cell heterogeneity within a cancer cell clone and its subclones as a major limitation to the immunohistochemical assessment of tumor cell proliferation.[1,6,7] Evaluation requires an arbitrary selection of tumor cell areas, and it cannot be determined with certainty whether these are representative.

Because of these numerous potential hazards in interpreting immunohistochemical data regarding tumor cell proliferation, a careful clinical follow-up is required in order to determine the value of formalin-resistant immunohistochemical proliferation markers as prognostic indicators of tumors. In two independent series encompassing 225 mammary carcinomas, a significant correlation between the proliferative activity as determined by Ki-S1 and clinical outcome could be demonstrated.[25,26] Therefore Ki-S1 seems to be suited for retrospective studies in order to establish the prognostic significance of proliferative activity of tumors using archival paraffin-embedded tissue samples.

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