Ki-67 detects a nuclear matrix-associated proliferation-related antigen

II. Localization in mitotic cells and association with chromosomes

R. VERHEIJEN1*, H. J. H. KUIJPERS1, R. van DRIEL2, J. L. M. BECK1, J. H. van DIERENDONCK3, G. J. BRAKENHOFF4 and F. C. S. RAMAERKERS1

1Department of Pathology, University Hospital Nijmegen, Geert Grooteplein Zuid 24, 6525 GA Nijmegen, The Netherlands
2Department of Biochemistry, University of Amsterdam, The Netherlands
3Department of Pathology, University of Leiden, The Netherlands
4Department of Electron Microscopy and Molecular Cytology, University of Amsterdam, The Netherlands

*Author for correspondence

Summary

In interphase cells the proliferation-associated antigen recognized by monoclonal antibody Ki-67 is almost exclusively located in the nucleoli. When cells at several stages of mitosis were examined for the localization of the Ki-67 antigen, a striking redistribution could be observed. During prophase the distinct nucleolar Ki-67 fluorescence changed to a bright irregular meshwork throughout the nucleoplasm. At metaphase the antigen appeared to be distributed in a reticulate structure surrounding the condensed chromosomes, while at late telophase a punctuated staining of the entire nucleoplasm was observed, which preceded the typical nucleolar localization pattern in each of the two daughter cells. Immunolabelling with Ki-67 of metaphase chromosome spreads revealed a circumferential

staining of the individual chromosomes.

The Ki-67 antigen is preserved in nuclear matrix preparations obtained after in situ fractionation of interphase cells. When mitotic cells were exposed to such treatments, the obtained fluorescence data suggested that the antigen may be part of the chromosome scaffold.

Quantification of the Ki-67 fluorescence signal using flow cytometry revealed the highest staining intensities in mitotic cells. Furthermore, it was shown that nutritionally deprived cells became negative for Ki-67.

Key words: nucleolar antigen, flow cytometry, confocal scanning laser microscopy.

Introduction

Ki-67 is a commercially available mouse monoclonal antibody that reacts with a nuclear antigen in proliferating cells. The antibody can be used to determine the growth fraction of human tumours in situ and has been shown to be of prognostic importance.

In a previous study we have shown that in interphase cells Ki-67 reacts with an antigen mainly localized in nucleoli (Verheijen et al. 1988b). Confocal scanning laser microscopy (CSLM) and immuno-electron microscopy on human MR65 monolayer cells revealed that this antigen is predominantly localized in the nucleolar cortex and in the dense fibrillar components. Furthermore, the Ki-67 antigen appeared to be preserved in nuclear matrix preparations obtained after in situ fractionation of MR65 cells in interphase. However, virtually nothing is known about the nature and biochemical characteristics of the antigen recognized by Ki-67. Despite many attempts we have not been successful in identifying the antigen in Western blotting or immunoprecipitation assays.

The present study was performed to examine the intracellular localization of the Ki-67 antigen in mitotic cells. In this respect it is shown that during mitosis the strongest immunohistochemical staining reactions with Ki-67 are obtained on the surface of the condensed chromosomes and that the antigen is preserved in 'nuclear matrix' preparations of mitotic MR65 cells. In our preceding study a profound heterogeneity of the Ki-67 staining intensities was observed between different cell lines and even within one cell culture. This phenomenon was further examined by flow cytometric (FCM) analyses of a cell line under variable growth conditions. Our findings are discussed in the light of recent data concerning Ki-67 expression levels in tumours.

Materials and methods

Methods
The indirect immunofluorescence assay, immuno-electron mi-
croscope, confocal scanning laser microscopy (Brakenhoff et al. 1985, 1989; Van der Voort et al. 1989), culturing of MR65 and MOLT-4 cells, as well as the preparation of in situ nuclear matrices of MR65 cells, were performed as described by Verheijen et al. (1988a). MOLT-4 cells were fixed and labelled for flow cytometry (FCM) essentially as described by Feizi et al. (1985).

Antibodies
Monoclonal antibody Ki-67 (IgG1; trade-name DAKO-PC, code no. M722) was purchased from DAKOpatents (Glostrup, Denmark) as tissue culture supernatant, and dialysed against 0.05 M-Tris-HCl, pH 7.2, and 15 mM-sodium azide. Other monoclonal antibodies used in this study were: 2.73, directed against the 70K U1 RNA-associated protein (Billings et al. 1982; Verheijen et al. 1980a,b); 41CC4, directed against the nuclear lamins (Burke et al. 1983; Verheijen et al. 1980a,b); and RKSE 60, directed against cytokeratin 10 (Ramakers et al. 1985). Since this latter protein is not expressed in MR65 cells or in MOLT-4 cells, this antibody was used as a negative control in the immunoﬂuorescence experiments.

The human autoimmune sera T5 and L66, both containing antibodies directed against nuclear proteins, were obtained from patients suffering from progressive systemic sclerosis (PSS). T5 has been described (Verheijen et al. 1986a), while L66 recognized several unidentified nuclear proteins.

FCM analysis
Cell analysis and sorting were performed using a cytofluorograph system SOH (Ortho Instruments, Westwood, MA, USA). Fluorochromes PI and FITC were excited by a 488 nm wavelength light beam from an argon ion laser (Spectra Physics, Mountain View, CA, USA). Fluorescence was measured simultaneously using a 515-530 nm filter and a longpass 630 nm filter for FITC and PI, respectively. All data were stored in correlated list mode on a PDP 11/34 (Digital Equipment Corporation, Maynard, MA, USA) for subsequent data analysis. Sorted cells were immediately collected on coverslips. From our FCM analyses with MOLT-4 cells it became obvious that it was necessary to obtain an optimal signal-to-noise ratio between positive and negative immunolabelled cells. For this reason it was important to select a suitable FITC-conjugated secondary antibody to mouse immunoglobulins. The FITC-labelled antibodies tested in this study included: (1) rabbit anti-mouse IgG F(ab')2-FITC from DAKOpatents, Glostrup, Denmark; (2) rabbit anti-mouse IgG-FITC from Nordic Immunological Laboratories B.V., Tilburg, The Netherlands; (3) goat anti-mouse IgG F(ab')2-FITC from Cappel, available through Organon Technika N.V., Turnhout, Belgium; (4) goat anti-mouse IgG (H+L)F(ab')2-FITC from American Qualex, La Mirada, CA, USA; (5) goat anti-mouse IgG (H+L)-FITC from TAGO Inc., Burlingame, CA, USA.

Chromosome spreads
Preparation of unfixed metaphase chromosome spreads of MR65 cells was performed essentially as described by Stemman et al. (1975). In summary, MR65 cells were treated for 2–4 h with 0.05% formalin (Sigma Chemical Co., St. Louis, MO) and incubated in hypotonic buffer (10 mM-Tris–HCl, pH 7.4, 40 mM-glycerol, 20 mM-NaCl, 5 mM-KCl, 1 mM-CaCl2, 0.5 mM-MgCl2) for 10 min at 0°C. Subsequently, the cells were spun onto glass slides using a Cytospin centrifuge (6 min, 1000 revs min⁻¹). The spreads were fixed and prepared for immunoﬂuor-

Fig. 1. Distribution of the Ki-67 antigen in MR65 cells in several stages of mitosis (A–F). DNA staining was performed with Hoescht 33258 (A'–F'). A. Interphase; B, prophase; C, metaphase; D, anaphase; E, telophase; F, late telophase/cytokinesis. ×1130.
scence microscopy as described for cultured cells (Verheijen et al. 1986b). Possible proteolytic activity present in the human antisera was reduced by using 0.5 mM-phenylmethylsulphonyl chloride (PMSC) during the first incubation step.

Results

Localization of the Ki-67 antigen in mitotic MR65 cells
To study the intracellular localization of the Ki-67 antigen in human cells we have used MR65, a human lung carcinoma monolayer culture in which the cells contain large nuclei and remain relatively flat during mitosis. The immunofluorescent localization of the Ki-67 antigen throughout the cell cycle of MR65 cells is displayed in Fig. 1. Staining of DNA in interphase cells with the fluorochrome Hoechst 33258 resulted in a diffuse fluorescence throughout the entire nucleus, except for the nucleoli (Fig. 1A'). The Ki-67 fluorescence in such cells appeared to be predominantly localized in the nucleoli as recently described (Fig. 1A; and see Verheijen et al. 1988b). When cells approach mitosis, the nucleoli gradually disintegrate and ultimately disappear as the chromosomes condense. At prophase, before this condensation has been completed, the distinct nucleolar Ki-67 fluorescence shifted towards a bright staining of a meshwork throughout the nucleoplasm, apparently associated with the chromatin (Fig. 1B). At metaphase a reticulum of interconnected fluorescent fibrils was often observed (Fig. 1C), changing into a more granular appearance during anaphase and telophase (Fig. 1D and E, respectively). During late telophase, when the nucleoli are reassembling, the Ki-67 fluorescence showed a speckled distribution pattern (Fig. 1F), followed by an aggregation of these speckles, thus restoring the distinct nucleolar fluorescence pattern in each of the two daughter cells.

Confocal scanning laser microscopy (CSLM) was applied to obtain more information about the precise, spatial localization of the Ki-67 antigen during mitosis. Fig. 2 shows the results of an optical sectioning procedure performed with this technique on two mitotic MR65 cells immunolabelled with Ki-67. The individual pictures correspond to optical sections all approximately 1 μm apart. Fig. 2A represents the plane nearest to the coverslip on which the cells were grown, while Fig. 2H represents the plane close to the top of the cell. These micrographs emphasize that all optical planes exhibit a similar fluorescence pattern of interconnected strands and clumps, apparently forming an intricate network within and around the condensed chromosomal material. Stereo images compiled from CSLM data consolidated this observation in several phases of mitosis, clearly showing an interchromatidous reticulum formed by the Ki-67 antigen (Fig. 3). Pseudocolour images (data not shown) emphasized the rather uniform staining intensity

Fig. 3. Stereo images of an MR65 cell in metaphase fluorescently labelled with Ki-67. The images were calculated from data obtained by confocal scanning laser microscopy. ×2600.
of the Ki-67 antigen in mitotic cells as compared to interphase cells (see also Verheijen et al. 1988b). The mitotic reticulum displayed small areas with a relatively high fluorescence intensity.

The results obtained using immunoelectron microscopy on mitotic cells were consistent with the observations in our immunofluorescence experiments. Fig. 4A shows an MR65 cell in prometaphase in which the Ki-67 staining reaction appeared to be the most intense at the periphery of the condensed chromatins. In mitotic cells from a human stomach adenocarcinoma a similar localization of the Ki-67 antigen was seen at the ultrastructural level (Fig. 4B).

To decide whether the Ki-67 antigen is associated with chromosomes, we have analysed metaphase spreads using immunofluorescence staining with several antibodies. In such preparations a bright fluorescence was observed with Ki-67 at the surface of all individual chromosomes (Fig. 5B). The monoclonal antibodies recognizing cytokeratin 10 (RKSE 60), the 70K U1 RNP protein (2.73) or the lamins (41CC4) did not stain metaphase chromosomes (Fig. 5E,G,I, respectively). However, using human autoimmune sera containing antibodies directed against various nucleolar proteins, similar chromosomal staining reactions were found with Ki-67 (Fig. 5M,O), indicating that the behaviour of the Ki-67 antigen during mitosis is not unique.

After in situ extraction of MR65 interphase cells with Triton X-100, a sodium deoxycholate/Tween-40 mixture, DNase I, RNase A and a high-salt solution (0.4-M ammonium sulphate), the Ki-67 antigen remained associated with the nucleolar residue in the obtained, so-called nuclear matrix-intermediate filament (NM-IF) scaffolds (Fey et al. 1984) (Fig. 6A; and see Verheijen et al. 1988b). However, when such preparations were obtained from mitotic MR65 cells the Ki-67 antigen appeared to be highly preserved as well (Fig. 6B–F), exhibiting similar distribution patterns throughout the various stages of mitosis as unextracted cells (compare Fig. 1). Staining with Hoechst 33258 of these scaffolds demonstrated that DNA was absent or barely detectable. Furthermore, SDS–polyacrylamide gel electrophoresis revealed that the histones were removed quantitatively from these structures (data not shown).

**FCM analysis**

In our immunofluorescence studies the Ki-67 fluorescence intensity in mitotic cells appeared to be higher as compared to interphase cells. Furthermore, striking differences in fluorescence intensity of interphase cells were observed between different cell lines and even within one cell culture (Verheijen et al. 1988b). To quantify objectively the fluorescence intensities in the several phases of the cell cycle we have performed FCM analyses of Ki-67-labelled MOLT-4 leukaemia cells, with RKSE 60 as a negative control. From these studies it became obvious that it was necessary to obtain an optimal signal-to-noise ratio between positive and negative immunolabelled cells. For this reason it was important to select a suitable FITC-conjugated secondary antibody to mouse immunoglobulins. In doing so we have monitored the levels of Ki-67 fluorescence signals in MOLT-4 cells during a culture period of 5 days, testing several FITC-conjugated antibodies. The results of this study are displayed in Fig. 7. The left column of graphs shows the relative fluorescence intensities of the total MOLT-4 populations, the middle column those of the G0/G1 fractions and the right column those of the G2/M fractions during the culture period. It is evident that the Cappel, TAGO and Nordic secondary antibodies show a
Fig. 5. Metaphase chromosome spreads (B,C,E,G,I,K,M,O) and interphase nuclei (A,D,F,H,J,L,N) of MR65 cells labelled with the monoclonal antibodies Ki-67 (A,B), RKSE 60 (D,E), 2.73 (F,G), 41CC4 (H,I), as well as with a normal human control serum (J,K), and the human autoimmune sera T5 (L,M) and L66 (N,O). C. A parallel staining with Hoechst 33258 of the same field as shown in B. ×1150.

relatively high background staining as compared to the DAKO and American Qualex (AQ) antibodies. Furthermore, the Cappel and TAGO antibodies exhibited a decrease in fluorescence in the control incubations with increasing culture time. Seen over this 5-day period the control signals of the DAKO, AQ and Nordic antibodies remained relatively stable, for the total cell population as well as for its $G_0/G_1$ and $G_2/M$ fractions. In all five secondary antibodies tested, the DAKO fluorescence intensity was found to be the lowest in the total MOLT-4 populations and in the $G_0/G_1$ fractions. Nevertheless, in our hands this antibody gave the best contrast, i.e. the lowest background (RKSE 60 signal) as compared to the Ki-67-dependent fluorescence signal. The difference in
staining intensity between the G2/M and G0/G1 fractions was also found to be the highest using the DAKO secondary antibody. The second best results were obtained using the AQ antibody. Considering the results mentioned above, we have performed all further FCM analyses with the FITC-conjugated secondary antibody obtained from DAKO.

Fig. 8 portrays a two-parameter FCM analysis of a 2-day culture of MOLT-4 cells after FITC-labelling using the antibodies RKSE 60, 2.73, Ki-67 and staining with PI. Fig. 8A depicts the profile of background staining obtained after labelling with RKSE 60. As demonstrated in Fig. 8B and C, the positive cell populations stained with 2.73 and Ki-67, respectively, are clearly discernable from the background of unlabelled cells. The 2.73 analysis pattern is a typical example of a profile commonly observed with several other nuclear and cytoskeletal antibodies. The profiles obtained by staining with 2.73 and Ki-67 of the cells in G0/G1 and S phase show a striking conformity and are almost superimposable. However, in contrast to observations with 2.73, the Ki-67 labelled G2/M cells display a broad range of FITC intensities with an extremely high positive fraction, suggesting the presence of two cell populations. In a study to obtain more information about the Ki-67-positive G2/M fraction (see Fig. 8C), these cells were separated into three subfractions by placing windows as indicated. The cells from each subfraction were sorted onto coverslips and the percentage of mitotic cells was estimated. Our preliminary data indicate that in the fraction with the highest Ki-67 fluorescence signal about 15% of mitotic cells were detected, whereas in the other subfractions almost no cells were found in mitosis. In contrast, the mitotic cells in G2/M fractions immunolabelled with antibody 2.73 were distributed equally over several subfractions. The lobed appearance of the MOLT-4 nuclei, however, made it very difficult to distinguish between mitotic cells and cells in interphase. When similar experiments were performed with HeLa cells, in which this problem did not occur, the observed distribution patterns were even more striking, i.e. the fraction with the highest Ki-67 signal contained 50-60% dividing cells, while in the other two fractions less than 10% of the cells were found to be undergoing mitosis. These results support the fluorescence data obtained in the monolayer cultures, in which the mitotic cells were also observed to display the highest Ki-67 fluorescence intensities.

In examining the effect of nutritional deprivation on the intensity of the Ki-67 fluorescence signal we cultured MOLT-4 cells for a period of 5 days without replacing the culture medium. Fig. 9 shows the FCM analyses of the Ki-67-labelled cells after 3 and 5 days in culture (Fig. 9B,D, respectively). Fig. 9A and C represent the corresponding background staining profiles. It is evident that in the 5-day culture a significant amount of cells in all phases of the cell cycle had become negative for Ki-67, a phenomenon not yet observed in the 3-day culture. When the cells were cultured at a higher concentration for 5 days this distribution pattern became even more pronounced, showing a large population of Ki-67 negative cells (Fig. 9F).

Discussion.

In a previous paper (Verheijen et al. 1988b) we reported...
that during interphase the antigen recognized by monoclonal antibody Ki-67 is localized predominantly in the nucleoli. To date, the Ki-67 antigen has not been identified in Western blotting or immunoprecipitation assays, probably because of the loss of its antigenicity during the experimental procedures used. As a result, insight into its possible function is lacking.

In 1983 it was shown by Gerdes and his co-workers (Gerdes et al. 1983) that during mitosis the Ki-67 antigen appeared to be associated with the chromosomes. The present study was developed to obtain more information about this association. Using immunohistochemical staining techniques we found the Ki-67 antigen to be localized around the condensed chromatin, often forming a reticular structure. To obtain better insight into the interaction of the antigen with the chromatin, selective extraction procedures in situ were applied to MR65 cells. For this purpose the cells were subsequently treated with detergents, DNase I, RNase A and a high-salt solution, resulting in so-called nuclear matrix–intermediate filament (NM–IF) scaffolds. Immunofluorescence studies of such preparations revealed a distinct staining of the nucleolar remnants in interphase nuclear matrices (see also Verheijen et al. 1988b), while 'matrices' of mitotic cells showed staining patterns similar to those observed in untreated dividing cells. Staining of these mitotic residual structures with Hoechst 33258 demonstrated that DNA was absent or barely detectable. Furthermore, SDS–polyacrylamide gel electrophoresis revealed that the histones were removed quantitatively from these structures. Although we cannot rule out the possibility that the Ki-67 antigen has been partly extracted, these results permit the conclusion to be drawn that during mitosis the integrity of the Ki-67 antigen distribution is apparently not dependent on the presence of DNA or histones. This suggests that the antigen is either associ-
ated with the non-histone proteinaceous structure known as the chromosome scaffold or is an integral part of it. Together with the results obtained in our preceding study (Verheijen et al. 1988b), these findings provide support for the concept that the chromosome scaffold constitutes the mitotic counterpart of the interphase nuclear matrix. Recently, we have scrutinized the current models concerning the structure of the chromosome scaffold in mitotic cells (Verheijen et al. 1988a). In one of the basic models that has been proposed this structure forms a rigid linear axial backbone in each chromatid (Paulson & Laemmli, 1977). Our observations with Ki-67 on metaphase chromosome spreads, in which the antigen was seen to surround the individual chromosomes, are not very compatible with such an internal structure enclosed by DNA and histones. A conceivable possibility, therefore, is that of a non-histone proteinaceous matrix localized at the periphery of the chromosomes in addition to an internal scaffold. Such a model of an external chromosome scaffold is supported by our confocal scanning laser microscopy data of intact mitotic cells, as well as by data obtained by several other investigators (Earnshaw & Heck, 1985; Hancock & Dessey, 1988), and have been summarized recently by Verheijen et al. (1988a). The possibility that the absence of staining of the internal regions of the chromatid is due to inaccessibility of the antigen by the primary and/or secondary antibodies in the highly condensed metaphase chromosomes cannot be excluded. However, this does not detract from our conclusion that the chromosome scaffold, if existing, is not a skeletal element merely present inside the chromatids, but is apparently also located at the periphery of the chromosomes.

In a previous report it was shown that the growth fraction of cell suspensions can be determined flow cytotmetrically after labelling with Ki-67 (Schwarting et al. 1986). This is of particular interest, since studies on human tumours have proven that the proliferative index as determined by Ki-67 immunostaining is an objective indicator of the biological behaviour of such neoplasms (Barnard et al. 1987). Recent investigations (Walker & Campion, 1988; Schutte et al. unpublished data), however, have shown that such a correlation does not exist for all tumour types. These authors could, for example, show tumour cases in which the number of cells positive for Ki-67, suggested as representing the total population of cycling cells, was significantly lower than the number of cells in S phase as determined by FCM. Our FCM results obtained with MOLT-4 cultures may explain these findings. Although present in S, G2 and M phase, a considerable number of these cells become negative for Ki-67 upon nutritional deprivation. These data are in accord with findings of Baisch & Gerdes (1987). It is well established that in solid cancers the central parts of tumour cell nests may become devoid of nutrients and oxygen. We anticipate that such a situation may result in a (partial) loss of Ki-67 antigen expression or detectability in these cells.
When comparing the Ki-67 data with those described for other proliferation markers (Mathews et al. 1984; Robbins et al. 1987) it is obvious that interesting similarities can be observed between the Ki-67 antigen and DNA topoisomerase II (Heck & Earnshaw, 1986). Recently, these latter authors have demonstrated that topoisomerase II, an enzyme that is involved in several aspects of DNA metabolism (reviewed by Wang, 1985), is a specific marker for proliferating cells. As well as this resemblance with the Ki-67 antigen, topoisomerase II is found associated with the interphase nuclear matrix as well as with the mitotic chromosone scaffold. Using a polyclonal antibody, the levels of topoisomerase II in interphase nuclei of both transformed chicken cell lines and an untransformed primary culture of chick embryo fibroblasts appeared to vary widely from cell to cell (Earnshaw et al. 1985), a phenomenon that we have also reported for the Ki-67 antigen in various cell cultures (Verheijen et al. 1988b). Moreover, mitotic cells and metaphase chromosomes are intensely stained by both anti-topoisomerase II antibodies and Ki-67. In positive interphase nuclei the anti-topoisomerase II antibody exhibits a punctate fluorescence pattern next to a bright diffuse staining of the entire nucleoplasm. So far, it is not known whether or not these fluorescent dots represent nucleoli and/or nucleolar bodies. Lack of biochemical data about the Ki-67 antigen prevents any further comparison between these two proliferation markers.

In summary, our results show that quantitative data obtained with Ki-67 in solid tumours should be interpreted with care. However, they also indicate that Ki-67 may become a strong tool in the study of cell-biological aspects of the interphase nuclear matrix organization and its mitotic counterpart, the chromosome scaffold.

We are indebted to Mr F. Rietveld (Nijmegen, The Netherlands) and Mr B. J. Mauw (Leiden, The Netherlands) for performing the immunoelectron microscopy. We also acknowledge the kind gifts of the monoclonal antibodies 41CC4 (from Dr G. Warren, Heidelberg, FRG), 2.73 (from Dr S. Hoch, La Jolla, CA, USA), and of the human autoimmune sera T5 and L66 (from Dr W. van Veenrooij, Nijmegen, The Netherlands).

This study was supported by the Netherlands Cancer Foundation, grant no. NUKC 1984-11, the Foundation for Fundamental Biological Research (BION), the Nijhakker-Morra Foundation and the Mauritius en Anna de Kock Foundation.

References


Verheijen, R., Kuipers, H., Voois, P., van Veen, W. & Distribution of the Ki-67 antigen 539


(Received 3 October 1988 – Accepted 16 December 1988)