Origin of microcells in the human sarcoma cell line HT-1080

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The aim of this study was to investigate the development of microcells in the human sarcoma cell line HT-1080 after interference with thiophosphamidum. We found that damaged interphase macrocells located at the projection of the nucleolus may form one or several microcells. The micronuclei of the microcells intensively incorporate the thymidine analogue 5-bromo-2′-deoxyuridine and strongly express argyrophilic nucleolar organiser region proteins. At an early phase of the development, the micronuclei contain fragmented DNA, but in subsequent phases, the micronuclei accumulate polymeric DNA, simultaneously with an increase in their size. After desintegration of the damaged macrocell, the microcells appear in the intercellular space. The microcells can enter mitosis and they strongly express the lung resistance protein. Electron microscopic observations suggest that coiled bodies are involved in the development of the microcells. Since the observed path of microcell formation differs from apoptotic cell fragmentation into apoptotic bodies, we propose a new term for this microcell development: *sporosis*. We suggest that self-renewal of the tumour stem cells is likely based on sporosis.

Keywords: Microcells, sporosis, immortality

1. Introduction

Microcells (small cells) are a natural component of tumour tissues. Microcells are distingnished by their roundish or oval form, scanty cytoplasm, and homogeneously and intensively stained nuclei. The relative numbers of microcells in tumour tissue markedly increase after chemotherapy, irradiation or immunotherapy.

Various ideas have been proposed for the origin of the microcells. Microcells in tumour tissue have been attributed to host immunocompetetive cell (macrophage progenitors, lymphocytes, etc.) and migration from blood capillaries into tumour tissue [21,24,48]. Other authors have considered microcells to originate from large damaged tumour cells undergoing programmed cell death (apoptosis) [20,41]. Apoptotic events are manifested by tumour cell shrinkage, nucleus condensation and fragmentation, internucleosomal DNA cleavage and formation of apoptic bodies (microcells) with low DNA content [6,16, 28,52]. Evidence also suggests that hypodiploid microcells develop from large tumour cells by multipolar mitosis [14] or by segregation of chromosomes in metaphase – blocked cytogenesis [29].

In previous investigations, we observed microcell development from perinuclear bodies of interphase nuclei in damaged macrocells of the Jungarian hamster fibroblastoma cell line 4/21 [9,10]. This path of microcell formation morphologically differed from cell death via apoptosis.

The aim of this study was to investigate the development of microcells in the established human sarcoma cell line HT-1080 after application of the cytotoxic drug – thiophosphamidum.

2. Materials and methods

2.1. Cell culture

The human sarcoma cell line HT-1080 [37] was maintained at 37°C in 25 cm^2 Karell flasks in Eagle medium supplemented with 10% bovine serum, glutamine and antibiotics (in air). The cells were passaged at 4 to 7 day intervals.

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2.2. Induction of microcells

Development of microcells was induced with thiophosphamidum. Cells grown in maintenance medium ($2–3 \times 10^5$ cells/ml) were placed in culture flasks. The cells were grown on coverslips at 37°C for 3–4 days. Then the medium was replaced with fresh medium containing thiophosphamidum in a concentration of 10 or 20 μg/ml. The cells were exposed to thiophosphamidum at 37°C for 24 h. The selective medium was than removed, the cells were washed 2× with Hank's balanced salt solution and fresh maintenance medium was added.

The cell samples for investigation were taken immediately after 24 h incubation with thiophosphamidum, and at 24, 48 and 72 h intervals after resupply with a fresh medium.

2.3. Light microscopy

The cells on coverslips were fixed in Carnoy's mixture (acetic acid : chloroform : 96% ethanol – 1 : 3 : 6) at 4°C for 10 min, washed in 70% ethanol, air dried and stained with methyl green-pyronine and azure-eosin. DNA was stained by Feulgen procedure.

2.4. AgNOR staining

The cells were fixed in Carnoy's mixture. One step AgNOR staining was conducted following the method described in Ploton et al. [34]. Staining mixtures were prepared *ex tempore* from one part of 2% gelatine solution in 1% formic acid and two parts of 50% silver nitrate solution. The cells were stained at 70°C for 5–10 min. The slides were than washed in water and subsequently in 5% sodium hyposulfite solution. Nuclear chromatin was counterstained by the Feulgen procedure.

2.5. Bromo-2′-deoxyuridine (BrdU) labelling and detection

BrdU is a thymidine analogue that can be incorporated into cellular DNA of cell cycle S-phase cells. Immunocytochemical assay was used for detection of BrdU incorporated into cellular DNA.

At the time of cell sampling, the culture medium from flasks was aspirated and BrdU-labelling medium (final concentration of BrdU – 10 μmol) was added. The cells were incubated with BrdU at 37°C for 60 min. The cover slips with the cells were washed with 0.1 M phosphate buffered saline (PBS), pH 7.2. The cells were fixed in 70% ethanol (in glycine buffer, 50 mmol, pH 2.0) for 20 min at −20°C.

Some of the cell samples after labelling with BrdU and washing with PBS were incubated in fresh maintenance medium at 37°C for 24, 48 or 72 h and then fixed.

Double-stranded DNA was denatured with 2 N HCl at room temperature for 20 min. The cells were neutralised with 0.1 M sodium borate buffer, pH 8.6. The cover slips were washed with PBS and the cells were covered with anti-BrdU monoclonal antibody working solution in PBS (1 : 100, pH 7.2) for 30 min at 37°C. The cells were washed with PBS and than incubated with anti-mouse-IG-AP, Fab-fragment solution (in PBS, 1 : 100, pH 7.2) for 30 min at 37°C. The cover slips were washed with PBS and cells were stained with colour-substrate solution in Tris-HCl-buffer (containing NBT and X-phosphate, and 5 mM levamisole, pH 9.2) at room temperature (RT) for 30 min. The cells and nuclei were counterstained correspondingly with methyl green-pyronine or by the Feulgen procedure.

Anti-BrdU mouse monoclonal antibody and anti-mouse-IG-AP, Fab-fragments, were obtained from Boehringer Mannheim, Germany.

2.6. In situ detection of DNA breaks

Two methods were used for *in situ* detection of DNA breaks: (1) terminal deoxynucleotidyl transferase (TdT)-mediated DIG-11-dUTP nick end labelling (TUNEL) method of Gavrieli et al. [19] and (2) holoenzyme of DNA polymerase-I (DNA Pol. I)-mediated DIG-11-dUTP incorporation into DNA nicks (nick translation) method of Oberhammer et al. [31]. TdT and DNA Pol. I were purchased from Boehringer, Mannheim.

The cells on cover slips were fixed at 4°C for 15 min in 4% paraformaldehyde solution, prepared in 0.1 M Tris/HCl buffered saline (TBS), pH 7.4, then washed in distilled water and air dried. The cells were incubated in 0.1 M TBS, pH 7.4, containing 0.025% Triton X-100 (Serva) for 30 min at RT, then washed with TBS, pH 7.4. The cells were deproteinated with proteinase K (10 μg/ml, 0.1 M TBS, pH 7.5) for 20 min at RT. Samples were pre-treated with the respective reaction buffer for 15 min at RT (TUNEL: 30 mM Tris/HCl, pH 7.4, 1% bovine serum albumin (BSA), 140 mM sodium cacodylate, 1 mM $CoCl_2$; nick translation: 50 mM Tris/HCl, pH 7.4, 1% BSA, 20 mM $MgCl_2$), then incubated for 1 h at 37°C in 25 μl of the respective

reaction buffer containing 1 μl d-NTPs (DIG DNA labelling mixture, Boehringer Mannheim) and the relevant enzyme (TUNEL: 7.5 U TdT; nick translation: 6 U DNA Pol. I). This reaction was stopped with 0.1 M Tris/HCl buffer, pH 7.4, containing 0.3 M NaCl and 30 mM sodium citrate (for TUNEL) or 50 mM Tris/HCl buffer, pH 7.4, containing 20 mM EDTA (for nick translation).

After extensive washing with 0.1 M TBS, pH 7.4, the incorporation of digoxigenin-labelled dUTP into DNA was detected using monoclonal anti-digoxigenin Fab-fragments (1 : 250, TBS, pH 7.4, 1 h at 37°C) linked directly to alkaline phosphatase (Boehringer Mannheim), followed by colour development with NBT/X-phosphate in Tris/HCl buffer containing 5 mM levamisole, pH 9.2, for 30 min at RT.

Positive controls were processed through proteinase K as described above and then treated with 1 μg/ml DNase I in 0.1 M TBS, pH 7.4, containing 1% BSA, for 20 min at RT. Negative controls were processed without any enzyme or without the anti-DIG-antibody. Reaction was not observed in negative controls.

The cells and nuclei were counterstained correspondingly with methyl green-pyronine or by the Feulgen procedure.

2.7. Lung resistance-related protein (LRP) detection

LRP protein was detected immunocytochemically using the p110-specific monoclonal antibody LRP-56, following the Scheper et al. [40] method.

The cells on cover slips were fixed with acetone for 10 min at RT and air dried. The samples were incubated with inactivated bovine serum (1 : 30 in 0.1 M TBS, pH 7.4) for 30 min at RT and than incubated for 45 min at 37°C with p110 specific monoclonal antibody – LRP-56 (1 : 100, TBS, pH 7.4). The cells were washed with TBS and then incubated with antimouse IG-AP, Fab-fragment solution (in TBS, 1 : 100, pH 7.4) for 30 min at 37°C. The cover slips were washed with TBS and cells were stained with colour-substrate solution in Tris/HCl buffer (with NBT and X-phosphate, 5 mM levamisole, pH 9.2) for 30 min at RT.

The cells were counterstained with methyl green-pyronine or by the Feulgen procedure.

The p110 – specific monoclonal antibody LRP-56 was kindly donated by Dr M.J. Flens, Department of Pathology, Free University Hospital, Amsterdam, The Netherlands.

2.8. Electron microscopy

Cell suspensions prepared by mechanical detachment of the monolayer were fixed with cold 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer pH 7.0 for 20 min and postfixed with 1% osmic acid solution for 1 h. The cell pellet was embedded in Epon. Ultra thin sections were contrasted by uranylacetate and lead citrate, and examined using a JEM-100 C electron microscope.

3. Results

3.1. General morphology

Mainly, two morphological types of cells were observed in the human sarcoma cell line HT-1080 exponentially growing in the maintenance medium on coverslips. Small, rounded, spindle-shaped or triangular cells were intensively stained with methyl green-pyronine or azure-eosin. The rounded cells usually showed many mitoses and the interphase cells contained modest sized irregularly shaped nucleoli. The second type of cells were large, polygon- or spindle-shaped, with one or more prominent nucleoli, and they stained pale with methyl green-pyronine or azure-eosin and rarely underwent mitosis. The large cell subpopulation was dominant in the monolayer. Many transitional forms between both cell types were also observed.

Microcell development was observed to occur in the large interphase sarcoma cells. Rounded associations of fine grained material in the nucleus of large cells were obvious. Usually, the association of granulae appeared at the projection of the nucleolus (Fig. 1A). These granules were strongly azurophylic. After staining with methyl green-pyronine, the granules could be classified into two groups: pyroninophilic and intensively stained with methyl green. Confluence of the granules stained with methyl green seemed to form micronuclei, with the pyroninophylic granules forming a narrow ring of cytoplasm around the micronucleus. As a result, microcells appeared to originate in the macronucleus at the projection of the nucleolus (Fig. 1B–D). In the subsequent period, microcell growth and their movement from the nucleolar or the nuclear projection to the cytoplasmatic projection of the macrocell was observed. One large tumour cell could potentially generate one or several microcells. The development of microcells was asynchronous. On

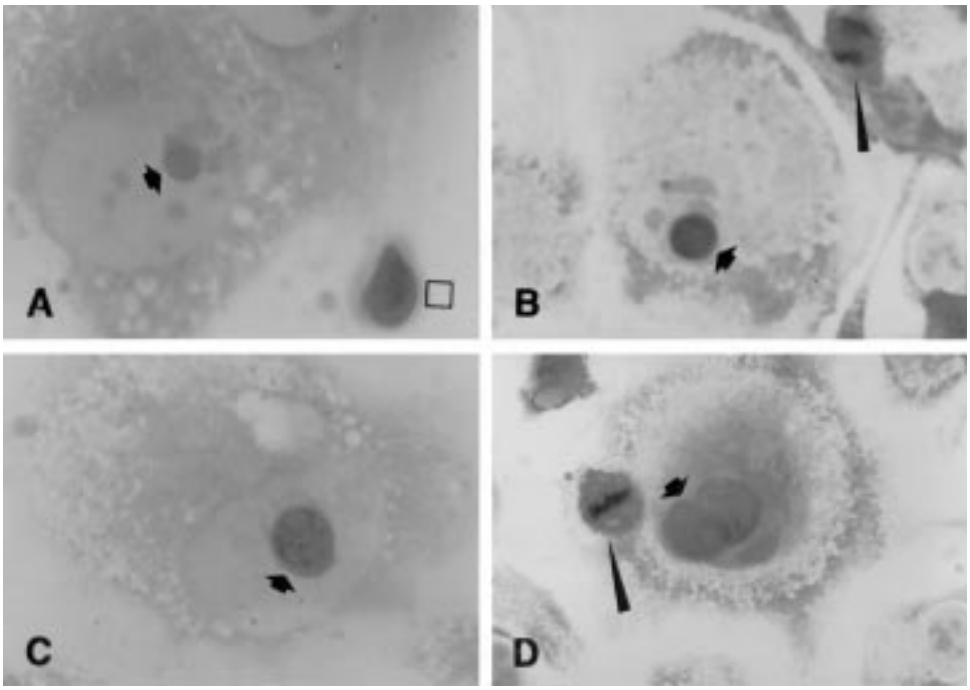

Fig. 1. Appearance of microcells in a macrocell nucleus. A – rounded collection of fine grain material forming the outline of a microcell in the macronucleus. B, C – a microcell in the macronucleus. D – a cluster of microcells in the macrocell. Thin arrows point to metaphase microcells. Thick arrows point to microcells in the macronucleus. Square – a microcell in the intercellular space. Methyl green-pyronine. Obj. 90×, oc. 7×.

the projection of a large tumour cell, several microcells were often seen in different phases of development. The microcells still associated with large tumour cells sometimes underwent mitosis (Fig. 1D). The large cells, during the period of microcell formation, usually preserved their integrity, however lysis of large cells was sometimes also observed. Some microcells often were seen in the necrotic debris of the macrocell. After degradation of the macrocells, microcells appeared in the intercellular space of the monolayer, and they joined the small cell subpopulation of HT-1080 cells (Fig. 1A).

Development of the microcells was observed both during cell cultivation in maintenance medium, especially on the 4th day or later, and during cell incubation with thiophosphamidum. In the latter case, microcell formation in the nuclei of macrocells was enhanced, especially after incubation with thiophosphamidum at a concentration of 10 μg/ml for 24 h and recultivation of the damaged macrocells in maintenance medium for 24–48 h. The appearance of microcells in macrocells usually was below one percent, but in specific cases of induction, the frequency of microcells associated with macrocells could reach a few percent. The large tumour cells, whether involved or not in microcell formation, usually underwent cellular lysis after incubation with thiophosphamidum. Some macrocells containing condensed nuclear chromatin or fragmented nuclei were also noticed. The microcells and the small cell subpopulation for the most part survived the thiophosphamidum treatment. During recultivation, the microcells became spindle- or triangle-shaped and increased in size.

3.2. AgNOR expression

The large-cell subpopulations usually were seen as a few coarse AgNOR dots in the nucleolar region of the macronucleus and as separate dots within the nucleoplasm. The small-cell subpopulations were visualized as many rather small AgNOR dots scattered within the nucleus, predominantly in the nucleolar regions.

Microcell development began as strong expression of many tiny AgNOR dots in small rounded or oval shaped outlines of the developing micronuclei in the macronucleus (Fig. 2A). In the developing micronuclei, strong AgNOR expression was associated with an accumulation of DNA. The Feulgen staining reaction of the developing micronuclei was very intensive. In the early phases of the micronucleus development,

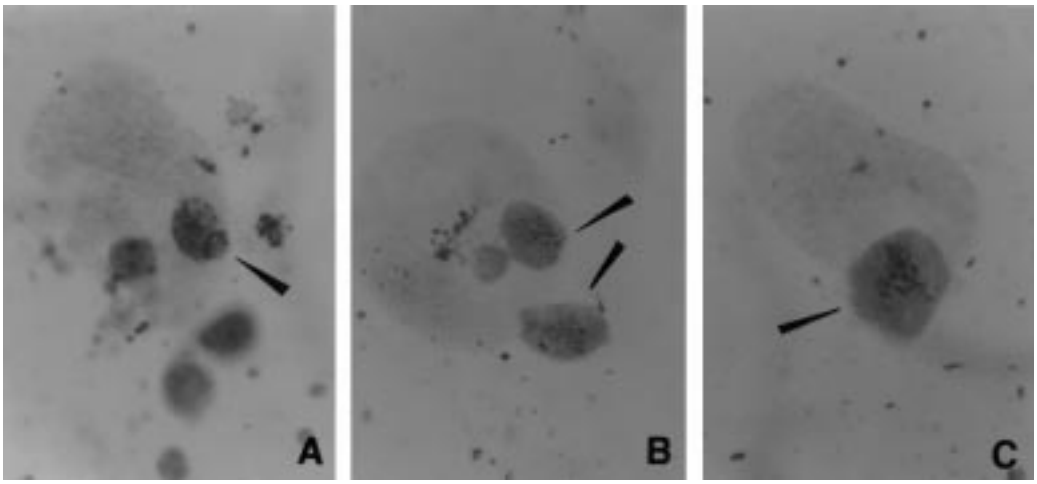

Fig. 2. AgNOR expression in micronuclei. A – AgNOR dots within an outline of a micronucleus. B – tiny AgNOR dots in the nucleoplasm of the micronucleus. C – strong AgNOR expression in the nucleolar region of the micronucleus. Arrows point to the developing micronuclei. NOR proteins were stained with silver nitrate, DNA was stained by Feulgen procedure. Obj. 90×, oc. 7×.

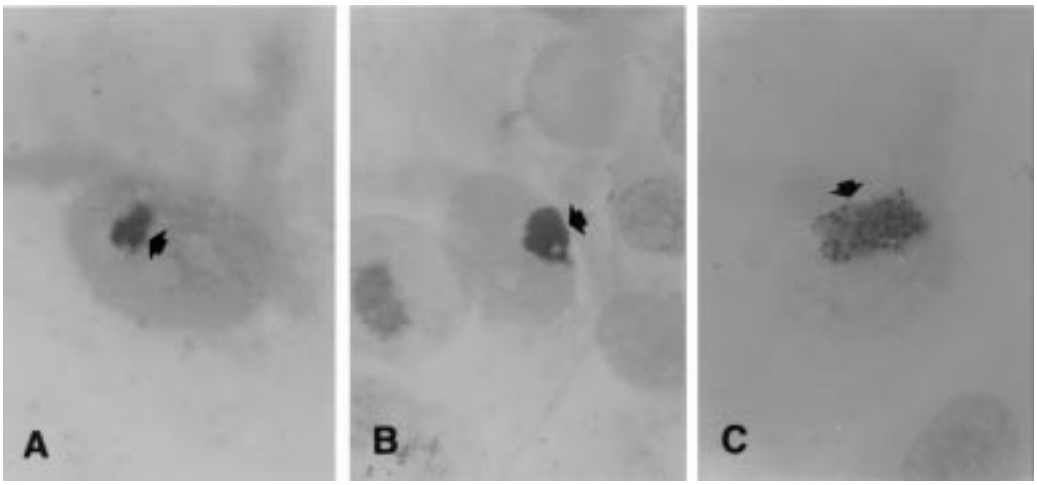

Fig. 3. Immunocytochemical staining of BrdU (blue colour) incorporated in DNA. A – stained developing micronuclei and unstained macronuclei. B – micronuclei in the late phase of the development were stained, while the macronuclei ghosts were unstained. C – BrdU mark dilution in the micronucleus 72 h after 1 h pulse labelling. Arrows point to the micronuclei. DNA was counterstained with Feulgen procedure. Obj. 90×, oc. 7×.

the AgNOR dots were usually scattered within nucleoplasm (Fig. 2B). After separation of the micronuclei from the remnants of macronuclei and the migration of microcells into the intercellular space, the AgNOR dots become coarse, concentrated around nucleolar regions of the micronuclei (Fig. 2C).

3.3. 5-Bromo-2′-deoxyuridine incorporation

The HT-1080 cells undergoing replicative DNA synthesis showed homogeneous immunocytochemical BrdU staining of the nuclei. Macrocells with developing microcells showed a speckled immunocytochemical staining for BrdU. The developing micronuclei were stained strongly, while the macronuclei were unstained. Intensive incorporation of BrdU into the developing micronuclei were observed during early to late (migration into intercellular space) phases of microcell development (Fig. 3(A, B)). When the developing microcells receiving pulse BrdU labelling were subsequently incubated in maintenance medium for 24–72 h, the BrdU staining of the micronuclei was weaker. After pulse BrdU labeling, the micronuclei

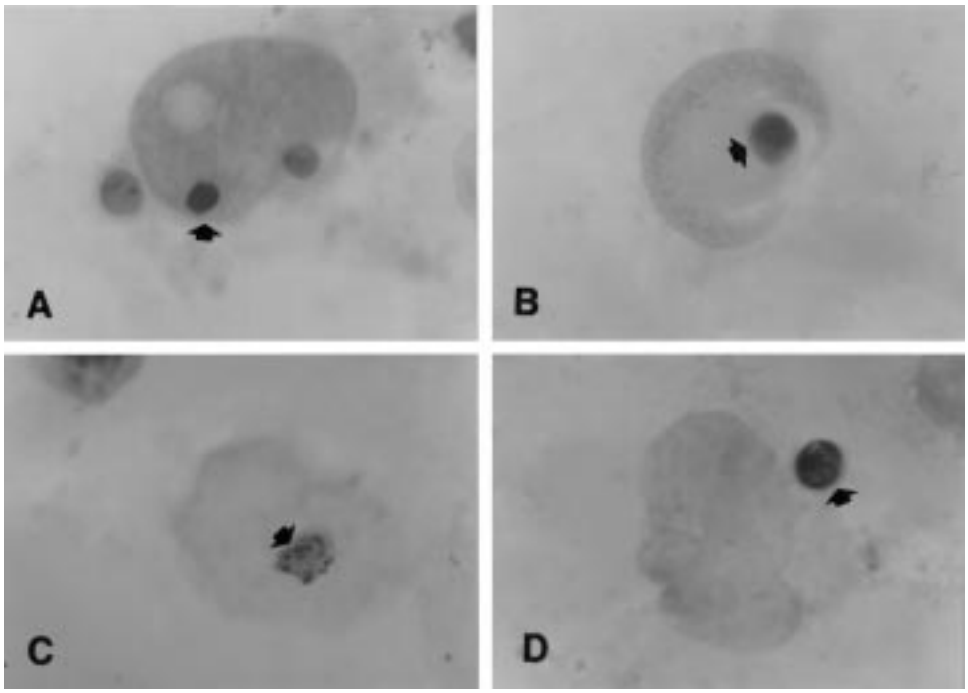

Fig. 4. Immunocytochemical staining of micronuclei for DNA strand breaks (blue colour) using TUNEL (A, B) and nick translation (C, D) method. A – outline of micronuclei shows strong TUNEL staining, while the micronuclei removed from the macronucleus are unstained. B – micronucleus show moderate staining with TUNEL method. C – the micronuclei outline shows weak staining by nick translation method. D – micronuclei removed from the macronucleus show strong staining by nick translation. Arrows point to micronuclei still associated with the macronucleus. DNA was counterstained by Feulgen procedure. Obj. 90×, oc. 7×.

showed strong and homogenously immunocytochemical staining (Fig. 3(A, B)). After subsequent incubation in maintenance medium, simultaneously with growth in size of the microcells, the immunocytochemical BrdU staining of the micronuclei become coarse granular and weaker (Fig. 3C).

3.4. Presence of DNA breaks

The micronuclei showed various staining patterns using TUNEL or nick translation methods, while the vast majority of macronuclei were unstained using either method. The strongest staining by TUNEL method was observed when the outlines of the micronuclei were forming before a significant concentration of DNA in it (Fig. 4A). With increasing DNA cocentration in the developing micronuclei and increase in micronuclei size, their staining with TUNEL gradually decreased and generally disappeared (Fig. 4(A, B)). Generally, immunocytochemical DNA 3′-OH end staining with TUNEL was more prominent in micronuclei still associated with macronuclei, compared with micronuclei removed from the macronuclei.

Nick translation weakly stained the outlines of micronuclei and the developing micronuclei still associated with the macronuclei (Fig. 4C). The micronuclei already detached from the macronuclei usually showed rather strong nick translation staining (Fig. 4D). Simultaneously with growth of microcells and their migration into the intercellular space, nick translation staining of the micronuclei decreased and later disappeared.

Some macronuclei showed a very similar staining pattern with TUNEL and nick translation methods, similar to the known apoptotic nuclei pattern. The sequence begins with a crescent-like staining pattern of the macronuclei around a nuclear envelope followed by an increase of macronuclei staining intensity, leading to staining of all macronuclei from the nuclear envelope to the central part. The sequence is ended by fragmentation of the macronuclei into variously sized, usually intensively stained bodies with rough surface (Fig. 5).

3.5. Expression of lung resistance-related protein (LRP)

HT-1080 cells, especially the large-cell subpopulation, generally showed weak cytoplasmic LRP stain-

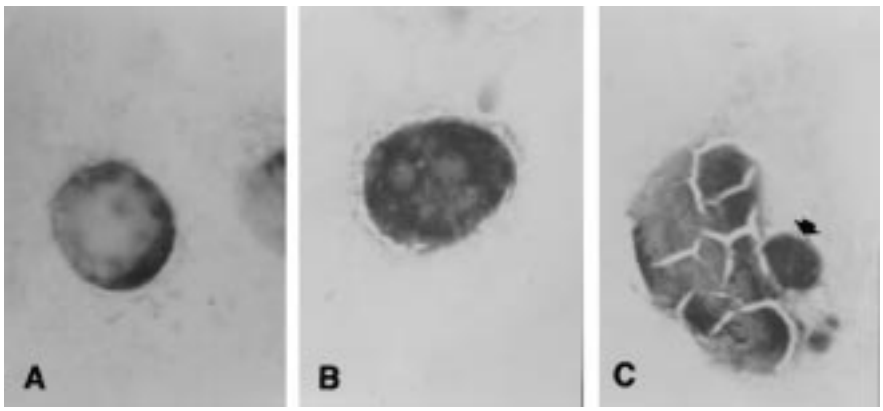

Fig. 5. Appearance of DNA strand breaks (blue colour) in apoptotic macronuclei stained immunocytochemically by nick translation. Macronuclei with crescent-like staining (A), total staining (B) and fragmented (C) macronucleus. Arrow points to an apoptotic micronucleus. DNA was counterstained by Feulgen procedure. Obj. 90×, oc. 7×.

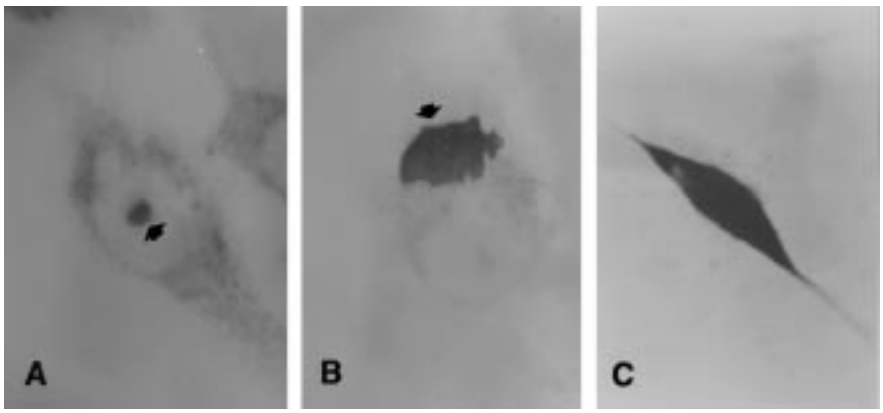

Fig. 6. Immunocytochemical staining of HT-1080 cells for LRP protein (blue colour). A – outline of a micronucleus in the macronucleus. B – microcell is still associated with the macrocell. C – microcell in intercellular space. Arrows point to developing microcells. Obj. 90×, oc. 7×.

ing. The immunocytochemical staining for LRP of developing microcells was strong and rather homogeneous. The developing microcells were usually vividly stained for LRP, in contrast to unstained macronucleus or the weakly stained cytoplasm of macrocells. The microcells in the intercellular space were also strongly positive for LRP (Fig. 6). Microcell staining for LRP protein gradually become weaker with their growth and transformation into spindle or triangular small cells.

3.6. Electron microscopy

The characteristic feature of HT-1080 cells damaged by thiophosphamidum was nuclear body development in the nucleolar region of the macronucleus (Fig. 7). The ultrastructural appearance of the nuclear bodies suggested that they may be coiled bodies. In the course of time, the nuclear bodies increased in size and moved toward the nuclear envelope (Fig. 8). The microcell

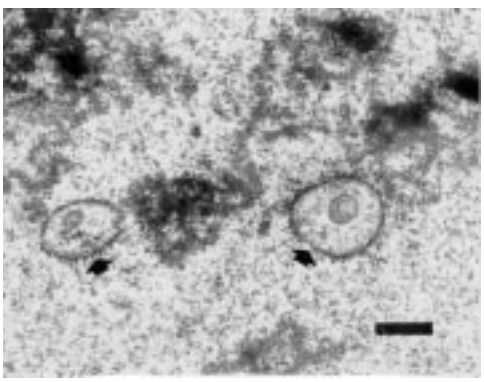

Fig. 7. Nuclear bodies (arrows) in the nucleolar region of a macronucleus. Bar 0.5 μm.

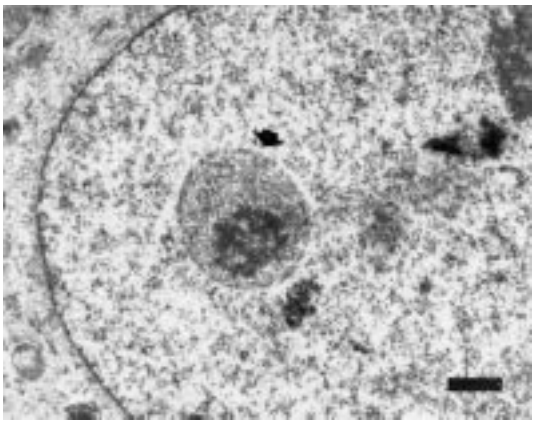

Fig. 8. Nuclear body (arrow) in the nucleoplasm of a macronucleus. Bar 0.5 μm.

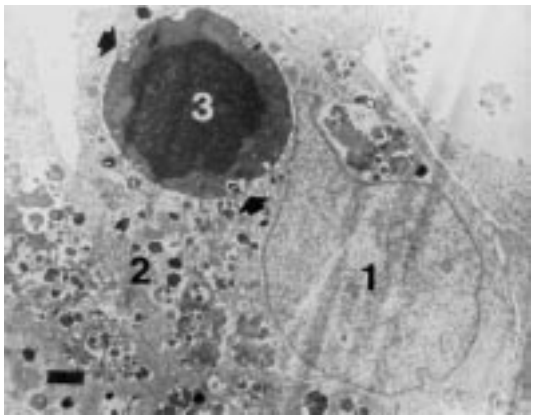

Fig. 9. Bud of a microcell in the cytoplasm of a macrocell. 1 – macronucleus, 2 – cytoplasm of the macrocell, 3 – microcell bud. Arrows point to sites of a microcell enclosing particles of the cytoplasm of a macrocell. Bar 1.0 μm.

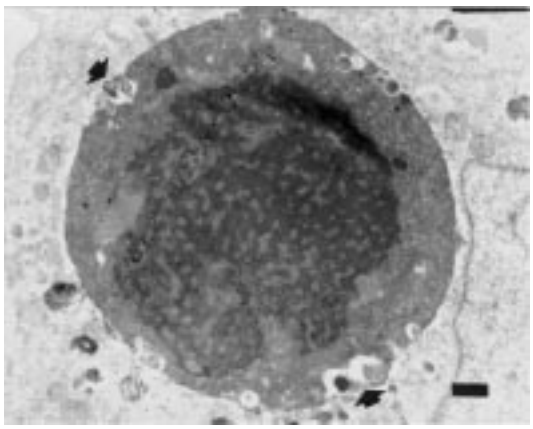

Fig. 10. Microcell bud in the cytoplasm of a macrocell. Arrows point to sites of a microcell enclosing particles of the macrocell. Bar 0.5 μm.

bud appeared at first in the cytoplasm of the macrocell. The microcell bud appeared round and with high electron density, especially in the central part (Fig. 9). The central part of the microcell bud also showed many small light areas that may correspond to AgNOR sites. Between the microcell bud and the cytoplasm of the macrocell, a narrow fissure (gap) lacking a limiting membrane was noticed. The periphery (future cytoplasm) of the cell bud enclosed particles of the surrounding cytoplasm of the macrocell (Fig. 10). The microcells in the intercellular space already showed distinct separated nuclei and cytoplasm. The cytoplasm of the microcells was observed to incorporate particles of the surrounding necrotic debris and to contain many phagosomes.

4. Discussion

Our light and electron microscopic observations suggest that microcells can develop from interphase macrocells. Interphase macrocells in early phases of microcell formation appeared to have a normal morphological structure. Only in the late phase of microcell development, the macrocells showed some swelling of the macronucleus, they gradually lost their stainability with basic dyes, and eventually degraded into amorphous debris characteristic for the cell death via necrosis. However, evidence suggested that the microcells migrated into the intercellular space. The observed morphological features of macrocells involved in microcell formation significantly differed from apoptotic fragmentation of macrocells into apoptotic bodies.

Programmed cell death (apoptosis) is manifested by cell and nucleus shrinkage and rounding, compactization and marginization of the nuclear chromatin, internucleosomal DNA cleavage, and nuclear and cytoplasmic fragmentation into membrane-bound apoptotic bodies (microcells) with low DNA content. Apoptotic events are gradually initiated in the whole (entire) nucleus: beginning at the periphery and ending in the central part of the nucleus [6,16,19,31]. Another feature of apoptosis is nucleolus dissolution [31], disruption [30], or dispersion [11] in both apoptotic nuclei and apoptotic fragments [31]. Apoptotic cells usually detach from the monolayer [43,49,50].

In contrast to macrocells involved in apoptosis, interphase macrocells involved in microcell formation are attached to the culture substrate and they exhibit well developed nucleoli and also AgNOR dots. Even

more, the origin of a microcell, which usually appears in the central part of the macronucleus close to nucleolus, is associated with strong expression of tiny AgNOR dots forming together rounded or oval outlines of the micronucleus. Generally, the expression of many small AgNOR dots scattered in the nucleoplasm are characteristic for the microcells.

AgNOR are nucleolus-forming rDNA loops associated with several argyrophilic proteins. The AgNOR protein quantity is related to high malignancy and rate of tumour cell proliferation [15,46]. Cancers with multiple, smaller, irregular and widely dispersed AgNOR in tumour cells have a more aggressive potential [42]. The level of AgNOR expression in HT-1080 cells indicated that the developing microcell subpopulations were metabolically more active and with a greater malignant potential than the macrocell subpopulations.

The suggested malignant capacity was confirmed by strong immunocytochemical staining of the developing microcells for Lung Resistance related Protein (LRP). LRP is a newly described p110-kD major vault protein involved in the active outward drug transport mechanism [39,40]. The LRP protein is closely associated with vesicular/lysosomal structures [40]. The deduced LRP amino acid sequence shows 87.7% identity with the 104-kD rat major vault protein. Vaults are multisubunit structures which may be involved in nucleocytoplasmatic transport [39]. Multidrug-resistant cancer cells frequently overexpress the 110-kD LRP protein. LRP overexpression in acute myeloid leukemia and in advanced ovarian carcinoma appears to be an indicator of poor response to standard chemotherapy and of adverse prognosis [3,26,39]. It seems that the developing microcells which strongly express LRP protein and AgNOR dots may be a natural multidrug-resistant subpopulation of the tumour cells which are able to survive the cytotoxic drug treatment.

The observation of BrdU incorporation into nuclear chromatin indicates that the micronuclei arose from newly synthesised DNA and that some DNA synthesis may occur in the presence of the inhibiter drug thiophosphamidum. The dilution of immunocytochemical staining of micronuclei during 1–3 days after pulse marking of DNA with BrdU suggests a rather long-lasting accumulation of DNA into the developing micronuclei and to a viability of the microcells for at least several days.

In contrast to our observations, apoptotic nuclear fragmentation or cell reaction to anticancer drug treatment or irradiation *in vitro* is very rapid process that usually affects about 30–50% of the cell population [33,44]. The transit time from cell shrinkage through total cell fragmentation into apoptotic bodies may last between 30 minutes to several hours [49]. Apoptotic bodies labelled with BrdU or [^{3}H]-thymidine may arise in cases of continuous or pulse cell exposure to BrdU or [^{3}H]-thymidine for several hours or days when cells labelled in S-phase are entering apoptosis [23,44,53]. However, a random distribution of BrdU in the nuclei and incorporation of BrdU inside micronuclei were also found after short-term incorporation of BrdU into apoptotic Friend leukaemia cells; the incorporation of BrdU into condensed chromatin was explained by DNA reparation processes [36].

The *in situ* detection of DNA breaks by TUNEL and nick translation methods showed that, in early phases of micronuclei development, they stained predominantly by TUNEL method, in the mid-phase of development the micronuclei stained predominantly by nick translation method, but in the late phases of development the micronuclei were unstained by either method. The TUNEL method utilizes terminal deoxynucleotidyl transferase, which catalyses the template independent polymerization of deoxyribonucleotides to the 3′-OH ends of DNA strands [19]. NICK translation method utilizes the holoenzyme of DNA polymerase-I, whereby one strand of DNA is used as a template to fill the breaks of single stranded DNA with polymerised deoxyribonucleotides [31].

The dynamic staining pattern for DNA breaks of the developing micronuclei can be explained as follows. The micronuclei in early stages of development, particularly in the outline-forming phase, were composed predominantly of low molecular weight DNA, presumably with oligodeoxyribonucleotides. The polymerisation of small DNA fragments into high molecular weight DNA which stains brightly by Feulgen procedure was indicated in subsequent stages of micronuclei development, simultaneous with their increase in size.

In apoptotic DNA cleavage, together with chromatin condensation within the whole macronucleus, sequential DNA depolymerization occurs initially from long chain (300 and 50 kbp) fragments to finally short chain (180–200 bp) fragments characteristic for extreme DNA degradation [12,16,45]. Our observations showed that apoptotic DNA degradation is accompanied by gradual loss of Feulgen staining of condensed and fragmented macronuclei.

An exclusive step of apoptosis is phagocytosis of dead cell fragments (apoptotic bodies) by neighbouring healthy cells in the cell monolayer or in the tumour tissue. The degradation of apoptotic cell fragments oc-

curs in phagolysosomes [6,41,43]. Our EM investigations showed, that the microcell buds are located directly in the cytoplasm of the macrocell without any contact with phagosomes. Moreover, the microcells incorporated particles of the surrounding cytoplasm of macrocells or necrotic debris. It seems that phagocytosis may be an important mode of feeding for the developing microcells.

Our ultrastructural observations suggested that the coiled bodies may be involved in development of the microcells. The coiled bodies are very dynamic intranuclear structures which often occur in association with the nucleolus, sometimes in direct continuity with nucleolar components. The coiled bodies contained splicing small nuclear ribonucleoproteins (snRNPs) which are subunits of spliceosomas, as well as a subset of the nucleolar proteins and the autoprotein p80 coilin [7,18,32]. The coiled bodies contain no detectable amount of DNA [47]. However, argyrophilic proteins [4,7,25] which are associated with rDNA loops involved in active synthesis of pre-rRNA have been discovered in coiled bodies.

The coiled bodies could serve as sites for initial preassembly and distribution of snRNP complexes (spliceosomes) for the three major RNA processing pathways: pre-mRNA splicing, pre-rRNA processing and histone pre-mRNA 3′ end formation [18]. Pre-mRNA splicing catalyses the removal of introns from nuclear pre-mRNA. Splicing snRNPs are associated not only with coiled bodies, but also with clusters of interchromatin granules and the interchromatin granule-associated zones [35]. The distribution of snRNPs in the nucleus is dynamic and may depend on cell type and functional state of the cell, as well as on its differentiation. SnRNPs are widely distributed throughout the nucleoplasm and show elevated concentrations in coiled bodies. SnRNPs often occur in a random intranuclear distribution, or they are predominantly located at the periphery of the nucleus where they form a spherical shell opposite to the nuclear envelope [38]. SnRNPs may be packed not only in large clusters of interchromatin granules in the nucleus, but also into membrane-associated structures at the nuclear periphery – snRNPs clusters inside a nuclear membrane [2]. The accumulation of splicing snRNPs and coiled bodies may also occur within the nucleolus [27,32].

Coiled bodies are especially prominent in the nucleoplasm of highly active cells [7] and in tumour cells [32]. The appearance rate of nuclear bodies in cancer cells may be associated with tumour invasive progression [51] and the ability of cells to enter apoptosis [8]. The formation of large bodies via coalescence of small bodies was observed in tumour cells long cultured [22] or arrested in S and G2 phases of the cell cycle [1].

We suggest a possible explanation for the transformation of the coiled bodies into microcells. At metabolic disbalance within the cell nucleus, caused by anticancer agents or depleted medium, the redistribution of snRNPs (spliceosomes) and formation of coiled bodies occurs in the damaged cells. Under conditions unfavourable for replicative DNA synthesis on chromosomes, the pre-mRNA associated with coiled bodies may obtain a new quality: pre-mRNA may begin to serve as a template for DNA synthesis.

The development of micronuclei begins with synthesis and accumulation of oligodeoxyribonucleotides within coiled bodies, followed by polymerization of oligodeoxyribonucleotides into high molecular weight DNA. Simultaneously with synthesis of oligodeoxyribonucleotides, rDNA loops pre-existing in coiled bodies are activated. The latter may be associated with development of cytoplasm in the microcells.

In the Jungarian hamster fibroblastoma cell line 4/21, we observed development of microcells from perinuclear bodies and nucleoli of damaged macrocells [9,10], and gave the name *sporosis* to this process. We consider now the perinuclear bodies to be one form of morphological expression of snRNPs clusters, i.e., inside a nuclear membrane [2]. The appearance of micronuclei (apoptotic bodies) in the perinuclear space was observed repeatedly [5,16]. We suspect that, in damaged tumour cells, various types of morphological pathways with different origin can lead to development of microcells. We propose to use the name *sporosis* to describe only those morphological pathways of microcell development which are associated with snRNPs clusters and disturbance of pre-mRNA splicing.

The above discussion leads to a new notion on the cytological mechanism of immortality of tumour cell populations. Up to now, the infinite life-span of malignant transformed cell lines and the regeneration of the tumours after anticancer agent application have been explained by cancer stem cell continuous self-renewal via mitosis [13,17]. Our data on microcell development from damaged tumour cells via sporosis suggests a new cytological mechanism of cancer cell population immortality (Fig. 11). Senescent or damaged tumour cells can die in the three ways: via necrosis, via apoptosis and via sporosis (Fig. 11-1). Necrosis or apoptosis finishes with full degradation of senescent or damaged tumour cells. In the course of sporosis in a damaged

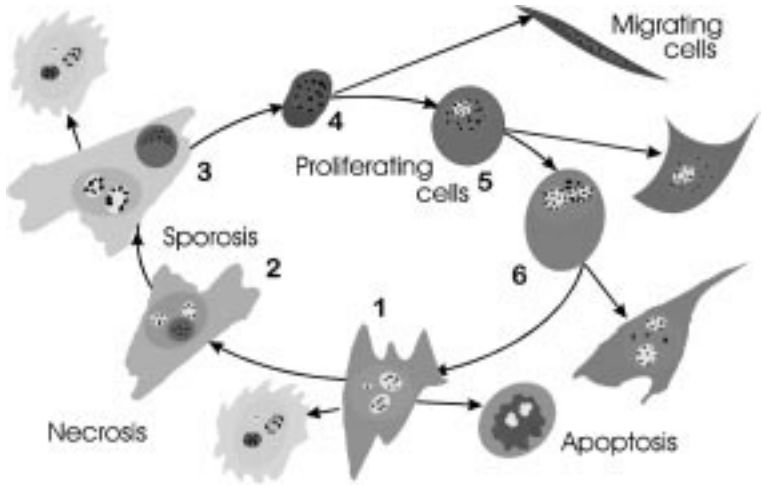

Fig. 11. A cytological mechanism of immortality of the human sarcoma cell line HT-1080.

tumour cell, disturbance of pre-mRNA splicing within the nucleus may lead to birth of a viable microcell(s) (Fig. 11-2). After desintegration of the mother cell, microcell migrate into the intercellular space (Fig. 11-(3, 4)). The microcells are metabolically very active and naturally resistant to anticancer agents. The microcells intensively accumulate nucleoproteins into their nuclei and cytoplasm. The rapidly growing microcells transform into young undifferentiated cells, beginning propagation via mitosis (Fig. 11-4). This means that stem cells can arise from microcells. The successive propagation of undifferentiated cells via mitosis leads to cell clone expansion and subsequent senescence. Anticancer agents facilitate the senescence of the proliferating cell clone (Fig. 11-(4–6)). The senescent tumour cells again are able to enter cell death via sporosis (Fig. 11-1).The vicious cycle of tumour cell life is now closed and the tumour cells are able to grow continuously.

The above described cycle of tumour cell life by sporosis allows us to explain the genetic instability of a tumour cell population and to provide new possibilities for anticancer drug search – the development of sporosis inhibitors.

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