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Suppression of Tumor Cell Susceptibility to Monocyte-Induced Cell Death by Growth-Inhibitory Signals Generated During Monocyte/Tumor Cell Interaction

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Abstract In a recently established serum-free in vitro system it has been demonstrated that the susceptibility of various human tumor cells to the induction of cell death by elutriated human monocytes is critically dependent on tumor cell density and growth state. In the present work it is shown by flow cytofluorometric analysis of bromodeoxyuridine incorporation rates and of expression of the proliferation-associated nuclear antigen Ki-67, that tumor cells forced out of the cell cycle into the quiescent state (G0), which can be accomplished by treatment with supernatant from monocyte/tumor cell interaction cultures, are no longer susceptible to the induction of cell death by monocytes. This suggests that processes essential for the lytic pathway cannot take place in quiescent cells. It is furthermore demonstrated that tumor cells are driven into G0 during interaction with monocytes and that the rate of transit from G1 to G0 increases with increasing monocyte dosage. This explains our earlier finding that maximum rates of tumor cell death are induced at rather low monocyte:tumor cell ratios of around 1:2 and that lysis is suppressed at higher monocyte dosages (van der Bosch et al.: *Exp Cell Res* 187:185–192, 1990). The potential significance of these findings for the supposed function of mononuclear phagocytes in tumor defense lies in the notion that tumor cells driven into G0 might escape this control and that signals involved in monocyte/tumor cell-interaction contribute to the accumulation of tumor cells in G0.

Key words: serum-free in vitro system, flow cytofluorometric analysis, bromodeoxyuridine, Ki-67, lytic pathway, cell cycle, cell death

The interaction of monocytes or macrophages with other cells frequently causes cytostatic and—in the case of tumor cells—also cytolytic effects [1–4]. Considering the ubiquitous presence of mononuclear phagocytes in the majority of the body tissues and the permanent influx of blood-borne monocytes into these tissues as well as their ability to produce a variety of growth-regulatory and tumor cytolytic cytokines [25–33] it does not seem unlikely that these effector cells are involved in growth control and tumor defense. The susceptibility to monocyte-mediated lysis varies greatly among different target cell species. Following interaction, usually a certain percentage of the target cell population survives. During subcultivation the survivors develop the same growth characteristics and pattern of lytic susceptibility as displayed by the original population. Repeated exposure of the survivors does not lead to the selection of inherently insusceptible subpopulations [5]. Thus, susceptibility to monocyte-mediated lysis is a feature not permanently expressed by all individuals even of clonal target cell populations. The requirements for the expression of *lysis* susceptibility by target cells have not been defined so far, although the susceptibility to macrophage-mediated *cytostatic* effects was observed by some authors to be limited to the G1 phase of the target cell cycle [6–8].

We have recently reported on experiments in a serum-free in vitro system which suggest that arresting growth and undergoing cell death

Abbreviations used: MO, monocyte(s); TC, tumor cell(s); SU, supernatant from MO/TC-co-cultures; BrdU, 5'-bromodeoxyuridine; PI, propidiumiodide; FITC, fluoresceine isothiocyanate; TNF, tumor necrosis factor; TGF, transforming growth factor; s.f.CM, serum-free culture medium.

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might be mutually exclusive responses of target cells to a monocyte challenge [14]. The data described in the present work support this view and show that especially cells leaving the cell cycle by transit into the quiescent state (G0) display a markedly diminished susceptibility to the induction of lysis by monocytes. The potential significance of these findings for the escape of tumorigenic cells from monocyte-mediated tumor cell destruction will be discussed.

METHODS

Cell Culture

The serum-free culture medium (s.f.CM) used for routine subculturing and for all experiments has been described previously [14]. It consists of a 1 + 1 mixture of DMEM + Ham's F12 buffered by 22 mM $NaHCO_3$ and 15 mM HEPES and is supplemented by insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor, EGF (5 ng/ml), triiodothyronine (0.1 nM), Na-selenite (75 nM), ethanolamine (8 μM), oleic acid (2.5 μg/ml), and fatty acid-free albumin (0.6 mg/ml). The clonal human tumor cell lines A375-2 derived from the A375 melanoma cell line and BT-B established from a surgical specimen of a bladder carcinoma in our laboratory have been also described earlier [14]. For passaging, cultures were washed twice with 10 ml of 0.9% NaCl/10 mM HEPES, pH 7.4 (NaCl/HEPES), and detached with 20 μg trypsin per ml NaCl/HEPES. Trypsinization was stopped with cold s.f.CM. Then cells were centrifuged and resuspended in warm s.f.CM for plating.

Preparation of Monocytes

Human monocytes were isolated from freshly drawn blood of healthy volunteers by Ficoll density gradient centrifugation and subsequent countercurrent centrifugation (elutriation) as described previously [14].

Monocyte/Tumor Cell Interactions

Freshly isolated monocytes were seeded onto confluent monolayers of A375-2 or BT-B cells, which were plated the day before onto collagen-coated 24-well plates (NUNC) at a cell density of 7.5×10^4 TC per cm^2, unless otherwise stated. The medium volume was kept constant at 2 ml per 2-cm^2 well and 50% medium renewal was performed daily.

Supernatant (SU) From MO/TC-Co-Cultures

Twelve $\times 10^6$ MO were seeded in 12 ml of s.f.CM onto 4×10^6 A375-2 cells plated 4 h before onto collagen-coated 58-cm^2 dishes; 24 h later, the supernatant was removed and centrifuged for 15 min at 2,000*g* and 0°C. The supernatant was filtered (0.22 μm), frozen in liquid nitrogen, and stored at −70°C until use.

Evaluation of Tumor Cell Numbers

For counting of tumor cells, the medium was aspirated and replaced by 1.5 ml 0.25% Triton X-100 in 0.4 mM EDTA (pH 7.5)/2-cm^2 well. Within 10 min under this condition, tumor cell membranes, as well as monocytes and their nuclei, are lysed quantitatively, whereas tumor cell nuclei are swollen and released as intact entities, which after 2 h of fixation with 0.5 ml 10% formaldehyde and transfer into 2 ml of 1.8% NaCl can be counted electronically as described previously [14].

BrdU Incorporation Rates

Direct immunofluorescent determination of the BrdU-incorporating TC fraction was performed following established procedures [9,12,13]. Briefly, at the times indicated in the diagrams MO/TC-interaction cultures were exposed for 60 min at 37°C to 50 μM BrdU by adding 1 vol.% of a 5 mM BrdU solution in s.f.CM. Thereafter the cultures were washed twice with 2 ml NaCl/HEPES per well and detached by a 5 min treatment with 200 μl of the trypsin solution described above. Following addition of 500 μl of ice-cold s.f.CM the single-cell suspensions were transferred to polystyrene tubes and centrifuged at 4°C for 5 min at 720*g*. Supernatants were only incompletely aspirated leaving about 50 μl of fluid with the cell pellets. These were resuspended in the residual fluid by tapping. Then, at room temperature, 1 ml 3 N HCl was added per tube under whirl mixing and the samples were incubated for 30 min at room temperature. After three washes with 0.5% Tween 20 in PBS and centrifugations at 720*g*, the pellets were resuspended in 50 μl of a 1:10 dilution in NaCl/HEPES of a FITC-conjugated monoclonal antibody against BrdU (Becton Dickinson) and incubated for 60 min at room temperature in the dark. After removal of the antibody solution and washing with 1 ml NaCl/HEPES the pellets were resuspended in 500 μl NaCl/HEPES for flow cytofluorometric analysis.

Ki-67 Expression

For indirect immunofluorescent detection of the expression of the proliferation-associated

nuclear antigen Ki-67, TC nuclei were prepared by exposure of MO/TC-interaction cultures to 1.5 ml 0.25% Triton X-100/0.4 mM EDTA (pH 7.5) at 0°C for 15 min. After adding 500 µl of ice-cold 10% formaldehyde in H_2O (pH 7.2) and incubation for 3 h at 0°C this suspension of fixed swollen TC nuclei was centrifuged for 5 min at 720*g* and resuspended in 0.05% Tween 20 in PBS (pH 7.4). In order to prevent clumping during the following procedures, the TC nuclei were then attached to the bottom of 24-well plates (NUNC) by 2 min centrifugation at 200*g*. After three washes with the above Tween 20 solution, 200 µl of a 1:500 dilution of the mouse monoclonal IgG1-antibody Ki-67 (ascites fluid) was added per well and the plates were gently agitated for 30 min at room temperature on a shaker. Then, after a further three washes (5 min each) as above, 200 µl of a 1:1,000 dilution of the FITC-conjugated second antibody was applied for 30 min. The second antibody was a $F(ab)_2$-fragment preparation of a goat antiserum against mouse IgG Fc-fragments (Dianova). The unbound second antibody was removed by three further washes as above. Finally, the wells were filled with 500 µl of the washing buffer. For detaching the TC nuclei from the bottom of the wells, plates were treated from below by ultrasound with a rod-shaped probe of 19 mm diameter at 50 W for 3 s each well. By this treatment suspensions of single nuclei were obtained, which were suitable for cytofluorometric analysis.

Propidium Iodide Staining

Propidium Iodide (PI) has been described as DNA binding stain suitable for the determination of cellular double-stranded DNA [9]. In order to avoid errors due to binding of PI to double-stranded RNA, usually an RNAse treatment precedes the PI incubation. However, Stevenson et al. have described [13] cellular RNA as no longer binding PI after the HCl treatment, which is used during the BrdU assay (see above). Furthermore, during preparation of TC nuclei for the Ki-67 assay most of the cellular RNA is removed with the cytoplasm. Thus these preparations can be used for double-labelling experiments with PI without prior RNAse treatment. PI staining was simply effected by adding 2% of a PI solution containing 2.5 mg PI/ml in PBS to the samples 1 h before cytofluorometric analysis.

Flow Cytometry

An Ortho cytofluorograph 50H was used, equipped with an argon laser operating at 250 mW and generating light of 488 nm wavelength. FITC fluorescence was detected by using an interference filter with a transmittance between 518 and 545 nm. PI emission was measured at wavelengths above 590 nm.

Sources of Substances

DMEM (Dulbecco's Mod. of Eagle's Medium), Ham's F12 medium, RPMI-1640 medium (Gibco, Karlsruhe). Insulin (bovine), transferrin (human), triiodothyronine, Na-selenite, ethanolamine, oleic acid, fatty-acid-free albumin, 5-bromodeoxyuridine (BrdU) (Sigma, Munich). Triton X-100, ethylenediaminetetraacetic acid (Na_2-salt) (EDTA), N-(2-hydroxyethyl) piperazine-N-2'ethanesulfonic acid (HEPES), (Serva, Heidelberg). Lymphocyte separation medium (Biochrom, Berlin). Epidermal growth factor (EGF) (Paesel, Frankfurt). Collagen (Vitrogen 100) (Collagen Corporation, Palo Alto). Formaldehyde (37%), (Merck, Darmstadt). Propidium iodide (Calbiochem, Frankfurt). FITC-labelled monoclonal antibody against BrdU (Becton Dickinson, Heidelberg). Ki-67 monoclonal antibody was generously provided by Dr. J. Gerdes, Forschungsinstitut Borstel.

RESULTS

In the present work we describe the population kinetics of two previously characterized [14] clonal human tumor cell (TC) lines—the melanoma A375-2 and the bladder tumor BT-B—under the influence of elutriated human monocytes (MO) in a serum-free in vitro environment as analyzed by electronic particle counting and flow cytometry.

Under a regimen of daily medium renewal, MO applied in appropriate quantity as a single dose to a confluent monolayer of TC cause a transient phase of cytolysis leading to massive TC loss between 24 h and 96 h following MO application (Figs. 1, 2). Thereafter, surviving cells slowly resume growth (Fig. 2). As reported previously [14], TC detaching from the culture substrate under the influence of monocytes were to more than 90% dead or determined to die. On the basis of their distinctive light-scattering characteristics it is possible by flow cytometry to analyze tumor cells in such interaction cultures separately from monocytes (Fig. 3A). Immuno-

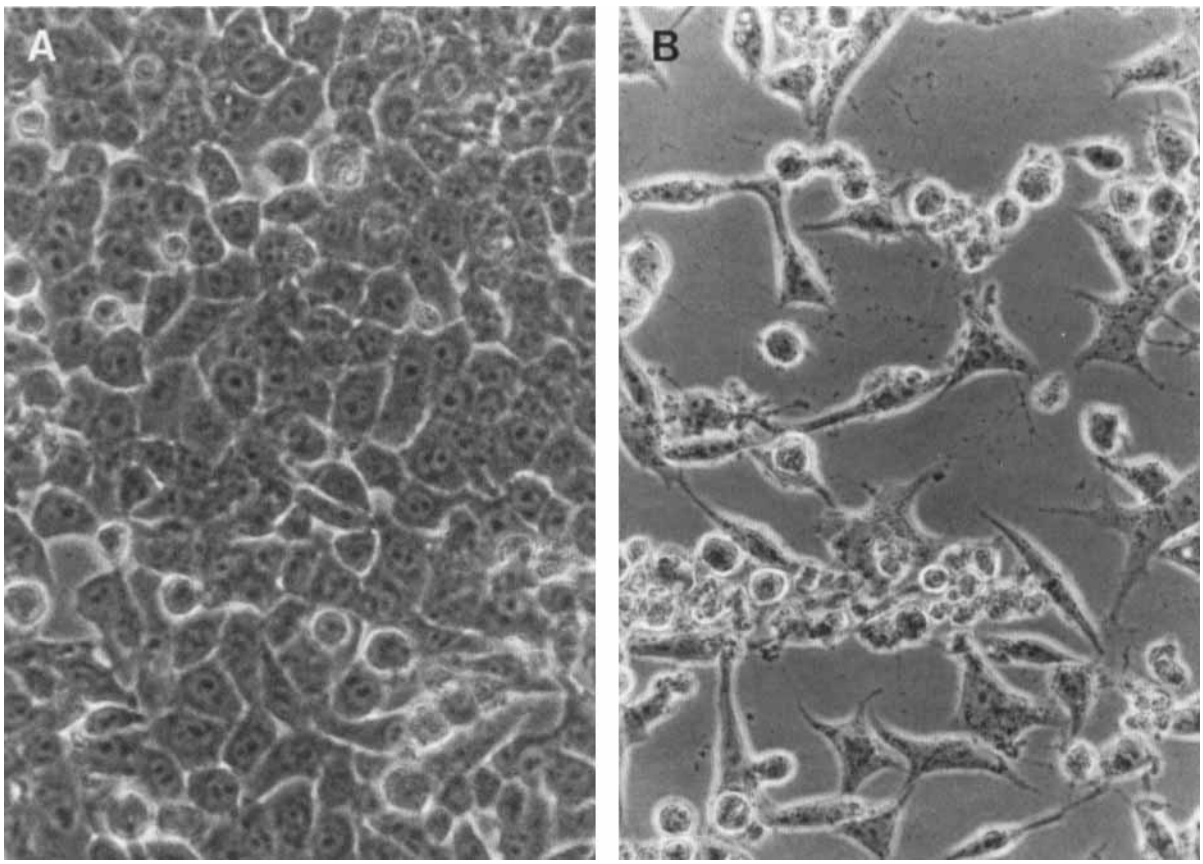

Fig. 1. Microphotographs. **A:** BT-B culture before exposure to MO. **B:** BT-B/MO-interaction culture, 72 h after adding MO. ~ × 300.

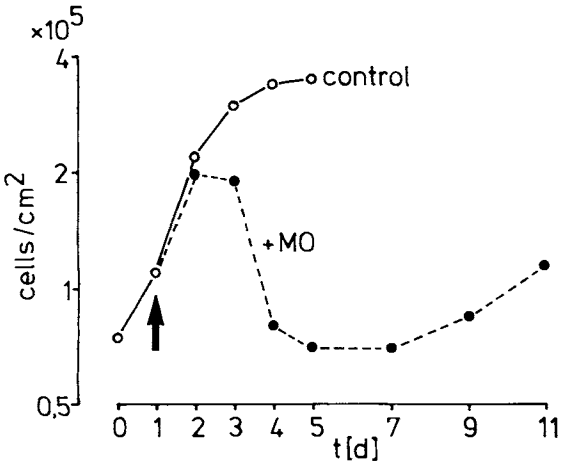

Fig. 2. Long-term development of BT-B cell number under the influence of MO as compared to untreated controls. At zero time BT-B cells were plated at a density of 7.5 × 10^4 cells/cm^2 onto 24-well plates (2 cm^2/well); 5 × 10^4 MO/cm^2 were added on day 1 (arrow).

fluorescent detection of 5-bromodeoxyuridine (BrdU) incorporation [9,12,13] has been used for determining the fraction of DNA-synthesizing TC as it changes under the influence of monocytes (Fig. 3B and Fig. 6, lower part). The Ki-67 antibody, on the other hand, which has been described as binding to a nuclear proliferation-associated antigen being expressed during all cell cycle phases but not by cells out of cycle in G0 [10,11], has been applied for detecting changes of the proliferative TC fraction. We have used this antibody here in conjunction with the DNA-staining propidium iodide (PI) for double labelling of suspensions of TC nuclei prepared from MO/TC-interaction cultures as described in the Methods section (*Figs. 4–6*). The assignment of cell cycle phases to subpopulations as detected by this method is demonstrated in Figures 4 and 5. An untreated control culture of BT-B cells displays a rather uniform (constant intensity) Ki-67 staining of G1, S, and G2 cells (Fig. 4A). After 48 h of MO exposure

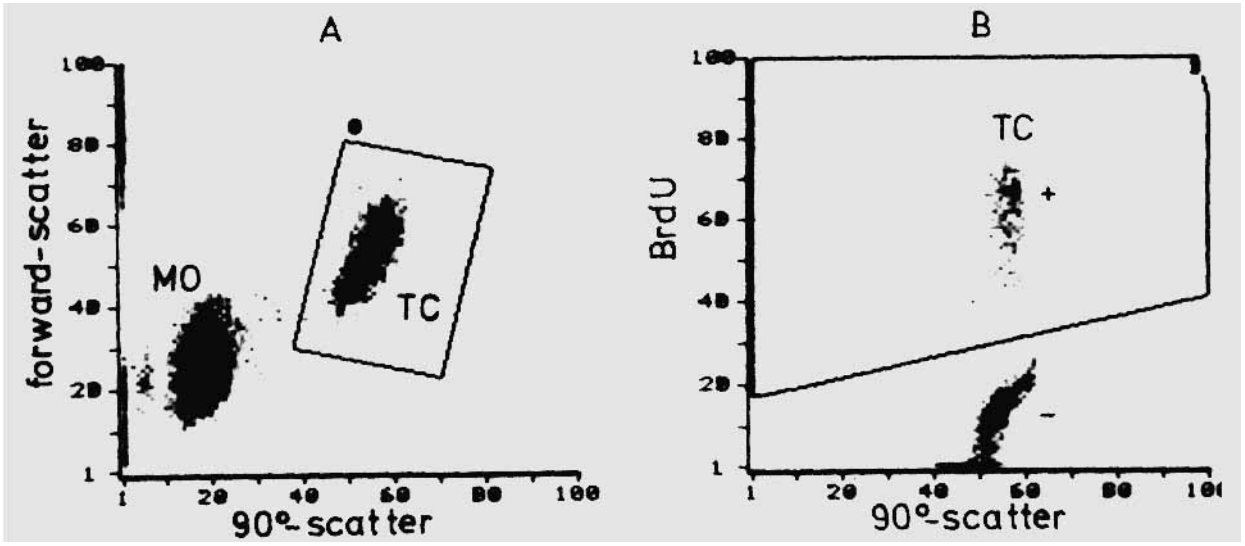

Fig. 3. **A:** Two-dimensional flow cytometric light-scattering diagram of a MO/BT-B-interaction culture 12 h after addition of 10^6 MO to 2 × 10^5 BT-B cells in a 2-cm^2 well, demonstrating the analytical separation of MO from TC. **B:** BrdU incorporation of the BT-B population described in A during a 1 h BrdU exposure 12 h after MO addition, as determined by direct immunofluorescent staining with FITC-labelled monoclonal antibody against BrdU, demonstrating separation of BrdU-positive from BrdU-negative BT-B fraction. Monocytes were gated out of the display on the basis of their distinct light-scattering characteristics.

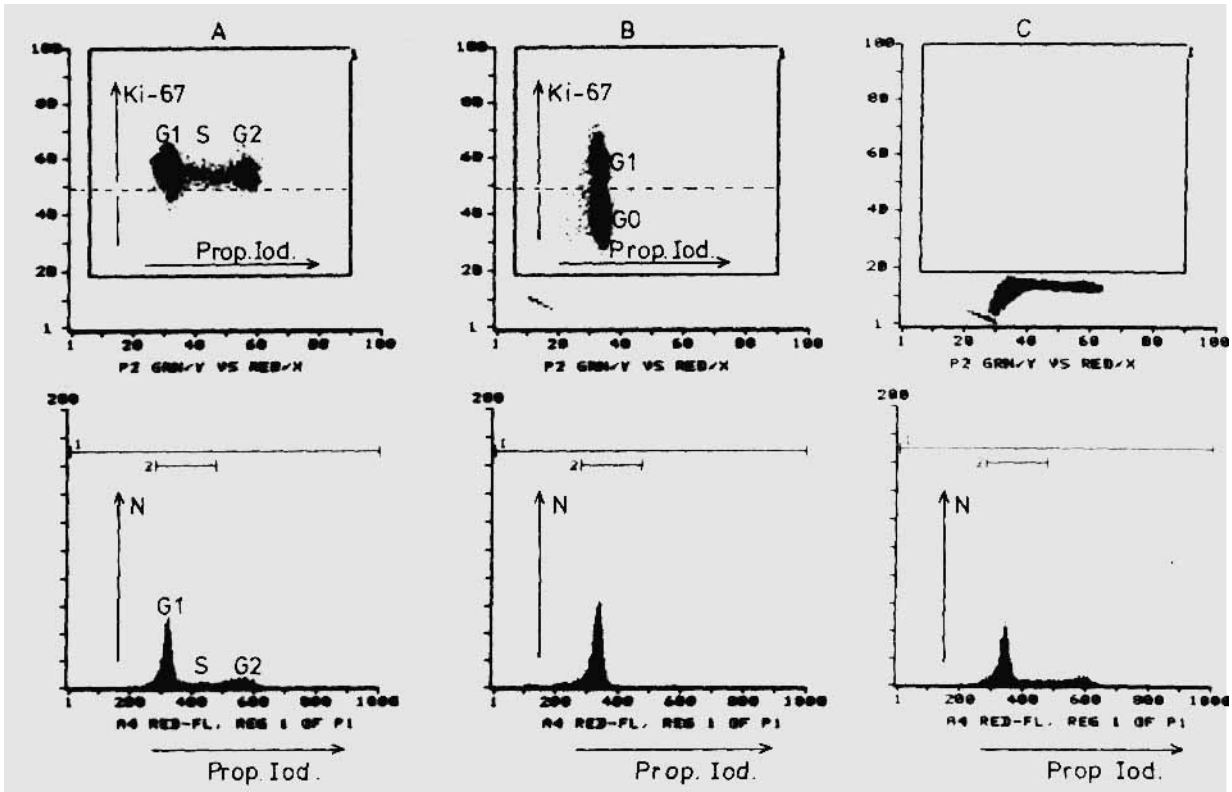

Fig. 4. Ki-67/PI double labelling. **Lower panel:** PI histogram, demonstrating cell cycle phase distribution. **Upper panel:** Two-dimensional Ki-67/PI cytogram. **A:** BT-B control population. **B:** BT-B cells after 48 h of low-dose MO exposure at MO:BT-B ratio of about 0.5:1, demonstrating the separation of the Ki-67-positive from the Ki-67-negative BT-B fraction. **C:** BT-B control as in A, but exposed to an unrelated first antibody (BO1) instead of Ki-67, demonstrating Ki-67 specificity.

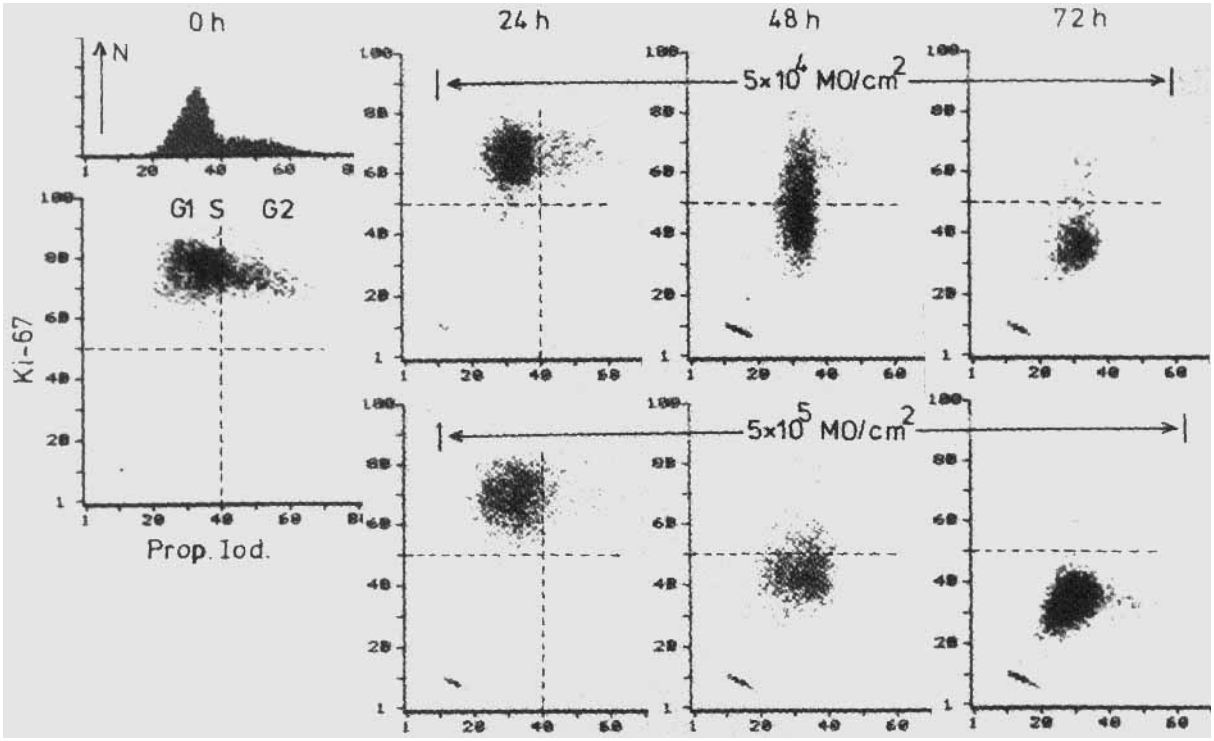

Fig. 5. Development of Ki-67/PI double labelling of A375-2 cells during 72 h of exposure to low (5×10^4 MO/cm^2) and high (5×10^5 MO/cm^2) MO doses. 0 h: A375-2 control before addition if MO.

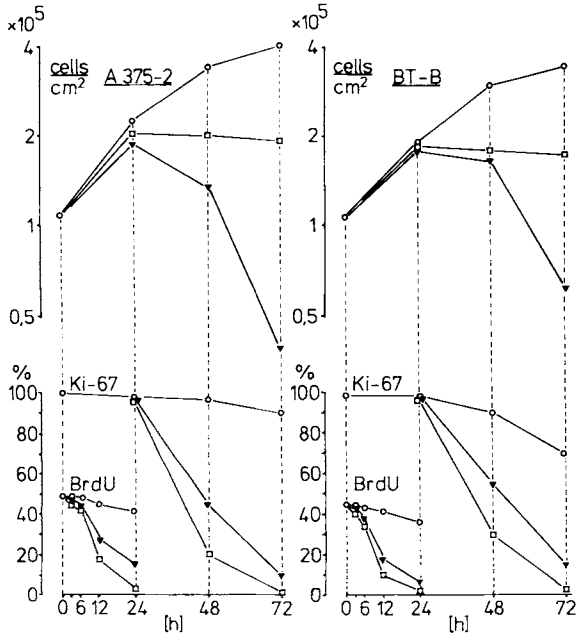

Fig. 6. Development of cell number (**upper panel**) and Ki-67-positive fraction and BrdU-positive fraction (**lower panel**) of A375-2 and BT-B cell populations. ○: without MO, ▼: with 5×10^4 MO/cm^2, □: with 5×10^5 MO/cm^2. MO were added at zero time to TC cultures seeded the day before; 50% medium renewal daily.

(Fig. 4B), no S- and G2-phase cells can be detected anymore. At this stage approximately 60% of the TC population displays a markedly lowered Ki-67 labelling at a propidium iodide position corresponding to the unreplicated DNA content of G1 cells, suggesting that these cells accumulate in G0. In Figure 5, the Ki-67/propidium iodide cytofluorogram of A375-2 cells is shown as it changes during 72 h of exposure to MO at two different MO:TC ratios. Significantly, under the low-MO dose (5×10^4/cm^2, corresponding to MO:TC = 0.5:1) disappearance of S and G2 cells at 24 h is less complete than under the high-MO dose (5×10^5/cm^2, MO:TC = 5:1). Likewise, the emptying of the Ki-67-positive compartment and the transit into the Ki-67-negative compartment during the following 48 h takes place at the low-MO:TC ratio more slowly than at the high one. These changes are quantitatively plotted in conjunction with the data on BrdU-incorporation rates and the simultaneous development of the TC numbers for A375-2 and BT-B in Figure 6. With both tumor cell lines, two reaction phases can be clearly distinguished:

I. During the first 24 h of MO exposure the BrdU-incorporating TC-fraction is dramatically

reduced from 45%–50% to about 5%–10%, while TC numbers increase almost unhindered like in controls. Cell death is a rare event in this reaction phase as revealed by microscopic examination. During this period the Ki-67-positive fraction remains unchanged at high levels near 100% like in control populations. At the end of this phase the majority of the TC population has accumulated in G1 (see Fig. 5). This accumulation proceeds more rapidly and more completely under the influence of the high-MO dose than under the influence of the low one.

II. The differences between the effects of low- and high-MO doses become more pronounced during the second phase of the interaction between 24 h and 72 h after MO application. Principally, two processes are observed during this period: 1) the size of the Ki-67-positive fraction is reduced (cells leave G1 via transit to G0). 2) The TC-number decreases via cytolysis. This latter process is especially pronounced under the influence of the low-MO dose leading to 60% to 80% cell loss during 48 h, whereas it is negligible in TC populations exposed to the high-MO dose. On the other hand reduction of the Ki-67-positive fraction (accumulation in G0) proceeds faster and more completely under the influence of the high-MO dose.

These results raised the question as to whether the failure to induce significant rates of TC lysis at high MO:TC ratios is due to the missing of induction signals or whether susceptibility to the induction of lysis is suppressed in TC driven into G0 with high rates under these conditions. Since it has been shown previously [14] that signals inducing lysis are produced at high MO:TC ratios even in higher amounts than at low MO:TC ratios, we favoured the hypothesis that a lowering of TC susceptibility at high-MO:TC ratios causes the failure to induce lysis under these conditions and that this state of lowered susceptibility is selectively expressed by cells driven out of the cell cycle into G0. An experiment supporting this view is described in the following.

For this purpose, BTB cells were exposed at sub-confluent cell density for several days to a supernatant (SU) harvested from MO/TC-interaction cultures as described in the Methods section. This procedure has been shown previously [14] to cause arrest of TC growth and concomitantly suppression of susceptibility to the induction of lysis by MO. In contrast, supernatants from TC or MO cultured alone without external stimulation by bacterial lipopolysaccharide and interferon gamma did not exert these effects [14]. In the present work a detailed analysis of the effects of SU treatment on cell cycle parameters and lytic susceptibility of the target cells was performed. Special attention was given to the question of to what extent these effects are reversible or irreversible. In Figure 7, early effects of SU treatment on the cell cycle of subconfluent BT-B cells are shown as revealed by dual-parameter cytofluorimetry following BrdU/PI labelling. The earliest effect detectable 3 h after SU application is depletion of early S-phase cells, as indicated by the arrow. At 24 h after SU application the majority of TC has accumulated in G1, with only few cells left in G2 and virtually none in S. The long-term development of cell number, BrdU-positive fraction, and Ki-67-positive fraction in these cultures is presented in Figure 8A for a time span of 8 days. In such cultures TC growth rate is slowed down under the influence of SU markedly and population doubling time is increased from about 1 day in untreated BT-B populations (e.g., control in Fig. 2) to 2–3 days. Furthermore, density-dependent saturation of growth takes place in the presence of SU at a cell density of $10^5/cm^2$, three- to fourfold lower than in untreated control cultures (e.g., Fig. 2). Concomitantly, continuous SU treatment leads to a lasting reduction of the BrdU-incorporating TC fraction to 5%–10% as compared to 45% in controls and to a continuous decrease of the Ki-67-positive fraction with a major drop during the first 2 days of SU exposure, indicating accumulation of the cells in G0. In order to analyse the effects of SU on BT-B cell growth competence and lytic susceptibility replicates of these cultures were detached on day 0 (untreated) and after 2, 4, and 8 days of SU treatment by trypsinization and re-plated at about confluent cell density in fresh culture vessels (Fig. 8B). Resumption of growth is increasingly suppressed in these reseedings with increasing duration of SU pretreatment, as evidenced by the growth curves (Fig. 8B: ○—○) of these populations. In parallel to Figure 8B, the developments of the BrdU-incorporating and Ki-67-positive fractions in these SU-pretreated populations are shown in Figure 8C: the BT-B population not pretreated with SU (0 days) displays high initial proportions of BrdU-incorporating (45%) or Ki-67-positive (95%) cells, which decrease as cell density increases and growth inhibition takes place. In contrast, SU-pre-

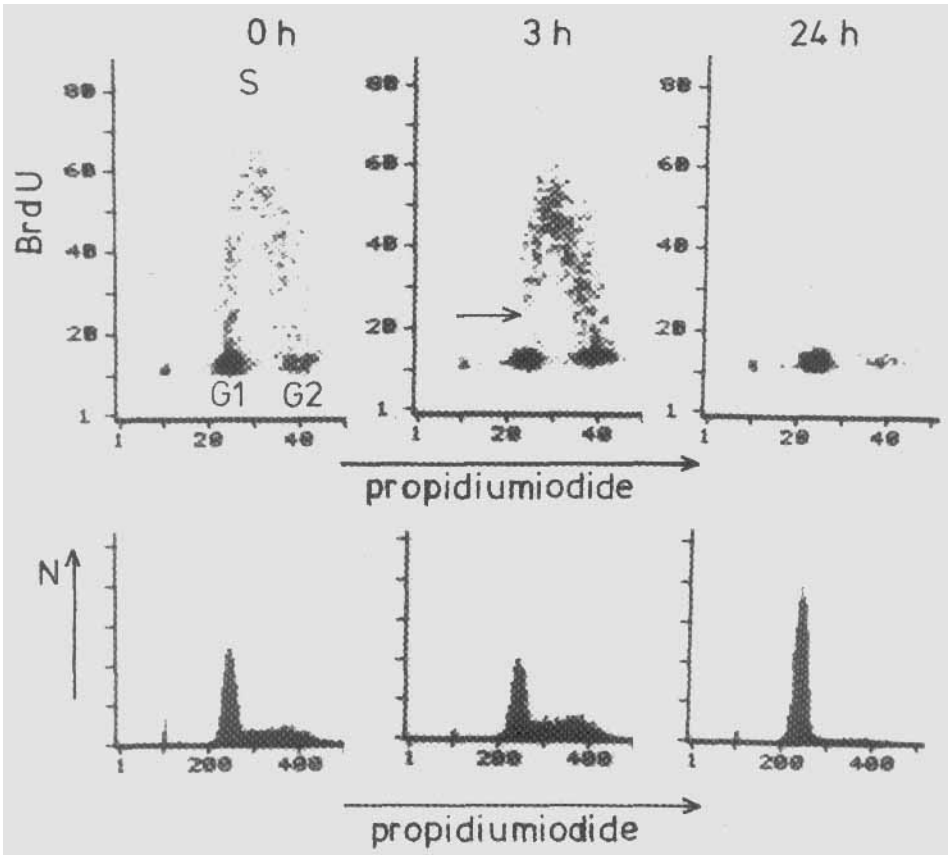

Fig. 7. Effect of SU on BrdU incorporation of BT-B cells 3 h and 24 h following application of 30% SU. 0 h: BT-B control without SU. At the times indicated (0 h, 3 h, 24 h), replicate cultures were exposed to BrdU for 1 h and thereafter prepared and labelled with PI as described in the Methods section.

treated populations (2 days, 4 days, 8 days) display low fractions of BrdU-incorporating cells, which increase only transiently to a minor extent. Also, Ki-67 expression recovers only transiently in BT-B cell populations pretreated for 4 days or 8 days with SU and starts ceasing again, when cells begin to saturate. In order to evaluate changes of tumor cell lytic susceptibility due to SU pretreatment, replicates of the reseeded populations were exposed to MO or SU at 3 h (arrow I) or 24 h (arrow II) following plating (Fig. 8B). Susceptibility to the induction of lysis by MO or SU decreases steadily with advancing SU pretreatment time and is especially low in reseedings of TC populations pretreated with SU for 4 or 8 days, as evidenced by the modest to negligible rates of lysis induced in these cultures. Recovery from the lysis-insusceptible state takes place with elapsing time after SU pretreatment concomitantly with rising Ki-67 expression. This is demonstrated by the somewhat higher rates of lysis induced by MO or SU application at 24 h (arrow II) as compared to the effects of application at 3 h following SU pretreatment (arrows I, Fig. 8B). In summary, SU pretreatment leads to increased capability of TC to perform a density-dependent negative growth control favouring TC transit to the Ki-67-negative quiescent state G0 and concomitantly renders TC insusceptible to the induction of cell death by MO or SU (Fig. 8B,C).

DISCUSSION

The susceptibility of tumor cells to the induction of cell death by signals generated during MO/TC interaction is an important parameter determining the outcome of such interactions. In the present work we show that the susceptibility of principally highly sensitive TC is markedly suppressed, when these cells are forced out of the cell cycle into G0. Furthermore, it is demonstrated that during MO/TC interaction TC are driven into G0 and that the surviving TC are G0 cells. These results suggest that pro-

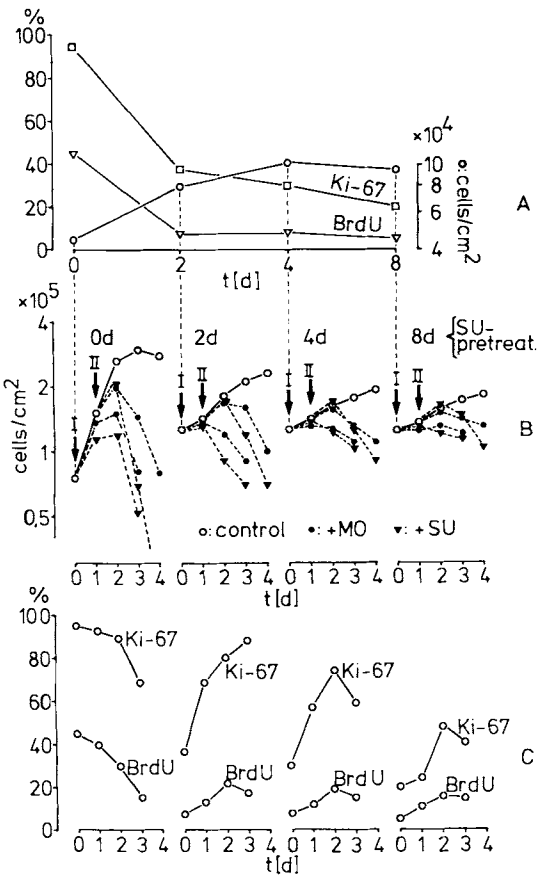

Fig. 8. Suppression by SU pretreatment of BT-B cell susceptibility to induction of lysis. **A:** Pretreatment of BT-B cells with SU. At zero time BT-B cells were seeded under 30% SU at a density of 4.5×10^4 cells/cm². Medium was renewed daily by 50% with fresh medium containing 30% SU. Cell number (○), Ki-67-positive cell fraction (□), and BrdU-positive cell fraction (▽) were determined at the times indicated. **B:** Exposure of SU-pretreated BT-B cells to MO or SU. At zero time, BT-B cells pretreated with SU as shown in A for 0, 2, 4, or 8 days were detached by trypsinization and re-plated onto fresh culture plates in s.f.CM. Their growth was recorded under these control conditions for 4 days (○—○). At the times indicated by arrows I and II (3 h and 24 h after plating respectively) replicates of the controls were exposed to a single dose of 5×10^4 MO per cm² (●) or to 30% SU (▼). They were counted for the 3 following days. Medium was renewed daily by 50% either with 100% fresh medium (○, ●) or with fresh medium containing 30% SU (▼). **C:** Ki-67 expression and BrdU incorporation of SU-pretreated BT-B cells following their reseeding in s.f.CM. The fraction of Ki-67-positive cells and the fraction of BrdU-incorporating cells of the control populations in B were determined for 3 days following the reseeding.

cesses essential for generating the lytic pathway usually cannot take place in G0. The rate of accumulation in G0 increases with increasing MO dosage. These results explain our earlier finding [14] of a maximum of tumor cell lysis rates at rather low MO:TC ratios of around 1:2 and of the suppression of lysis at higher MO dosages.

The fact that tumor cells have accumulated in G1 after 24 h of exposure to MO without significant rates of lysis and that lysis takes place at maximum rates during the following 48 h in the absence of a significant S/δ2-fraction suggests that cells die preferentially in G1 and that the ability of a cell to undergo transition from G1 to G0 under the influence of MO is critical to its escape from suffering lysis under this condition. The ability to escape into G0 is more pronounced in cells having been exposed to SU as evidenced by their density-dependent reduction of Ki-67 expression at cell densities at which untreated control cells display undiminished Ki-67 expression (Fig. 8B,C). Thus treatment with SU seems to enable these tumor cells—at least transiently—to perform a density-dependent inhibition of growth associated with accumulation in G0 at about confluent cell density, mimicking normal cell behaviour.

It seems that in principle cells subjected to an MO challenge have two possibilities to respond to the signals generated during this interaction: the first step is in each case the accumulation of the target cell population in G1 without significant cell loss. The second step is either transit from G1 to G0 or, if this does not happen fast enough, triggering of the lytic program in G1. As shown in the present work, the rate of accumulation in G0 is increased with increasing MO dosage, thereby preventing lysis. Previously it was shown [14] that the efficiency of blocking G1/S transit in subconfluent TC cultures increases with increasing MO dosage too. Low MO numbers could only incompletely prevent G1/S transition, suggesting that under conditions of maximum rates of lysis (low MO numbers) preparations for G1/S transit are not suppressed significantly and that target cells performing preparations for G1/S transit express the state susceptible to the induction of lysis.

These results once more support our hypothesis [14] that during MO/TC interaction the decision on whether a target cell will die or not is made on the basis of its capability to respond to the monocyte challenge by arresting its cell cycle appropriately or not. This decision takes place in the target cell and represents an efficient selection mechanism for the elimination of cells not complying with the laws of negative growth control: potential tumor cells. With this hypothesis there is no need for a kind of frequently

postulated [e.g., 36] receptor/ligand-based recognition process on the side of the effector cell as an instrument for selection. Tumor cells can escape the presently proposed selection mechanism if they mimic normal cell behaviour as demonstrated above. If this selection mechanism is effective in vivo, it means that during establishment of spontaneous tumors selection takes place for the survival of cells capable of switching between transformed and untransformed phenotypes, as exemplified here with BT-B and A375-2 cells.

The identity of the signals mediating the phenomenology described here has not been determined yet. TNF-alpha probably does not play a major role in the present system, since neither BT-B nor A375-2 cells show a lytic response to recombinant human TNF-alpha, nor were antibodies against TNF-alpha effective in preventing the induction of TC lysis by MO or SU at concentrations which completely abolished L-cell lysis under otherwise identical conditions [14]. However, interesting parallels to the present observations on MO/TC interaction have been reported in the literature concerning the action of TNF in other cellular systems. It has been shown [15] that TNF, causing growth inhibition in endothelial cells, induces in these cells the transcription of the competence gene AP-1/c-jun, a transcription factor involved in the mediation of stimulation of DNA synthesis in quiescent cells by serum and growth factors [16–19]. Moreover, in the presence of FGF, a growth factor promoting G0/G1 transit in endothelial cells, TNF induces endothelial cell death [20]. Similarly, it has been reported that the sensitivity of L cells to TNF-mediated lysis is proliferation associated [21]. In post-confluent quiescent Balb 3T3 cell cultures it has been observed that a wave of cell death precedes the onset of DNA synthesis induced by TNF at concentrations between 10 and 100 ng/ml [22]. These authors concluded that cell death occurred in a certain G1/G0 subpopulation. They furthermore showed that a phospholipase activity of the target cells was essential for mediating the growth-stimulatory as well as the cytolytic effect of TNF. All these observations suggest a coupling between the signal transduction processes leading to cell death and to cell proliferation and support the concept that the decision on whether to die or to divide is made when preparations for G1/S transit are in progress, depending on the instantaneous levels of growth-inhibitory and growth-stimulatory signals [14]. A further interesting example on the same line is the involvement of TGF-beta in the induction of programmed cell death in the rat prostate following androgen deprivation by castration [23]. In this case it has been shown that cell death is accompanied by the expression of the otherwise proliferation-associated genes c-fos and c-myc [24].

Mononuclear phagocytes are capable of producing a variety of cytokines with sometimes multifunctional potencies, including TNF-alpha [25], TGF-alpha [26], TGF-beta [27,28], interferon-alpha [29], interleukin-1 [30], interleukin-6 [31], fibroblast growth factor (FGF) [32], and platelet-derived growth factor (PDGF) [33]. Several of these cytokines can also be produced by tumor cells [e.g., 34,35]. Taking into account the foregoing examples it seems likely that the induction of TC-death by MO as described in the present article is a consequence of general homoeostatic principles, which can be realized by various individual signal combinations, depending on the signal generation, reception, and transduction potentialities of the target cell.

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