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Biochimica et Biophysica Acta 1721 (2005) 98–106
BIOCHIMICA ET BIOPHYSICA ACTA
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# Low tumor cell density environment yields survival advantage of tumor cells exposed to MTX in vitro

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Received 18 August 2004; received in revised form 10 October 2004; accepted 13 October 2004
Available online 26 October 2004

**Abstract**

Stable resistance to methotrexate has been well characterized after prolonged treatment of the HT-29 colon cancer cell line, but the mechanism of cell survival at the early stages of the drug resistance process still remains unclear. Here, we demonstrate that human cancer cells in vitro are sensitive to methotrexate only above a critical cell culture density, which specifically coincides with their ability to deplete the extracellular nucleosides from a fully supplemented culture medium. At lower cell densities, extracellular nucleosides remain intact and allow salvage nucleotide synthesis that renders cells insensitive to the drug. Consistently, medium conditioned by cells seeded at standard cell densities sensitizes low cell density cultures. Extracellular nucleosides are the determinants of sensitivity because the latter effect can be mimicked with the use of inhibitors of nucleoside cellular import and reversed by supplying exogenous thymidine and hypoxanthine. Interestingly, treatment at a sensitizing cell density does not preclude the survival of less than 1% of the cells—which have no intrinsic resistance—owing to the inability of the dying cell population to condition the culture medium; this population thus survives indefinitely to continuous treatment by keeping adapted to a low cell number. This cell density-dependent adaptive process accounts for the initial steps of in vitro resistance to methotrexate (MTX) and provides a novel mechanistic insight into the cell population dynamics of cell survival and cell death during drug treatment.


*Keywords:* Drug resistance; Neoplasm [G12.392.395]; Tumor cell; Cultured [A11.251.210.770]; Cell survival [G04.335.316]; Nucleoside [D13.570]; Neoplasm; Residual [C04.697.700]

## 1. Introduction

Methotrexate (MTX) is an antimetabolite drug that reversibly binds to and inhibits dihydrofolate reductase (DHFR), an enzyme involved in the production of reduced folate needed for the synthesis of thymidilate and purines [1]. Owing to the requirement of deoxynucleotides in DNA synthesis, MTX can impair tumor cell growth and induce cell death by secondary genotoxic effects or apoptosis [1,2]. Combination therapies including MTX are currently used to treat several tumor types, including osteosarcomas and lymphomas, among others [3]. However, MTX is less effective against many other tumor types: in the case of colorectal cancer, for example, the tumors are generally resistant; in the case of head and neck squamous cell carcinoma, a proportion of tumors are initially responsive but rapidly become resistant to MTX therapy [3].

Two general mechanisms have been proposed to understand how drug resistance emerges in tumors that are initially sensitive to drug treatment. First, tumor cell

*Abbreviations:* DHFR, dihydrofolate reductase gene; FBS, fetal bovine serum; IC50, 50% inhibitory drug concentration; LCD, low cell density; MTX, methotrexate; NBMPR, nitrobenzyl-mercaptopurine ribonucleoside; DP, dipyridamole; SCD, standard cell density

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doi:10.1016/j.bbagen.2004.10.006

subpopulations with decreased susceptibility to drug-induced apoptosis may be previously selected under conditions of hypoxia, low pH, or nutrient deprivation, possibly through acquired somatic mutations in genes such as Tp53 [4]. This type of drug resistance occurs as a tumor progression event, and resistant cells are inherently present as a subpopulation at the beginning of drug treatment. Second, drug resistance may emerge by acquisition of permanent changes in a tumor cell subpopulation while antitumor treatment is administered. This phenomenon has been called acquired resistance, and it arises de novo owing to the genetic instability of cancer cells—e.g., by gene amplification [5–7]—or to epigenetic modulation of gene expression—e.g., by overexpression of genes involved in drug resistance, such as multi-drug resistance genes [8,9]. In both mechanisms, the resistant cell subpopulation will be selected to outgrow as a recurrent tumor during the treatment. In this context, a number of tumors displaying resistance to MTX, as well as resistant cell line variants obtained by in vitro treatment, contain genetic alterations indicative of acquired resistance. These alterations include DHFR gene amplification, decreased cellular uptake by mutations in the reduced folate carrier, and mutations in the DHFR gene itself, among others [10]. Opposed to acquired resistance, the so-called adaptive resistance to MTX and other antimetabolite drugs can be attained provided that an extracellular source of nucleosides is available [1]. These extracellular nucleosides allow salvage nucleotide synthesis that bypasses the effects of drug-inhibited de novo synthesis [11–14].

The HT-29 colon cancer cell line has provided important clues on the processes occurring during acquisition of MTX resistance. HT-29 cells treated in vitro with 0.1 μM MTX undergo massive cell death during the first week of treatment [15,16]. Survival of a minor cell fraction leads to the emergence of stably resistant populations only after continuous treatment for several weeks [15]. HT-29 cell populations selected at MTX doses higher than 0.1 mM appear to acquire resistance by amplifying the DHFR gene locus [16] and by overexpressing the folate binding protein α [17]. However, the mechanism involved in the acquisition of resistance to 0.1 μM MTX is still unclear. First, it has been proposed that some HT-29 clones are selected by MTX treatment to give rise to the cell population stably resistant to 0.1 μM MTX. This notion is based, in part, on the fact that this stably resistant population displays differentiated features only found in <10% of the cells in the untreated cell line [15]. Accordingly, clonal selection has been clearly demonstrated at the highest doses of HT-29 resistance to MTX: amid the cell population resistant to 10 μM MTX, only those cell clones with enterocytic differentiation features and with the ability to amplify the DHFR gene are selectable by 1 mM MTX [18]. Alternatively, a second mechanism has been proposed with which some sort of adaptive process precedes the acquisition of stable resistance to 0.1 ìM MTX, so that it would explain the lag phase before stable resistance shows up in vitro.

We have been interested in studying the initial steps of resistance in the HT-29 model. First, we found that the parental HT-29 cell population does not contain, prior to treatment, cell subclones selectable by 0.1 μM MTX. Since the resistance displayed by the cell population surviving to the few first weeks with MTX is reversible, then an adaptive mechanism must account for their survival. When analyzing the cell population dynamics during this adaptive process, we found that sensitivity to MTX is strictly dependent upon the cell density. This phenomenon reflected the ability of the cell population under treatment to deplete the extracellular nucleosides from the culture medium. In this manner, HT-29 cell cultures below a "critical cell density" are insensitive to MTX because the cell number is too low to deplete the extracellular nucleoside content. Importantly, despite initial sensitivity at the time of treatment in standard density cultures, HT-29 adapt to a low cell number by means of cell death; this condition coincides with the "critical cell density" and allows sustained, yet reversible, resistance. These results thus define a transient mechanism of adaptation preceding the acquisition of stable resistance to 0.1 μM MTX.

## 2. Materials and methods

### 2.1. Cell culture

MTX was obtained as a 2.2 M solution (Almirall, Spain); stocks were prepared in culture medium at 10 mM and stored frozen until used. Dipyridamole (DP) and nitrobenzyl-mercaptopurine ribonucleoside (NBMPR) were purchased from Sigma (St. Louis, MO). Unless otherwise indicated, cells were routinely seeded in 20- or 57-$cm^2$ cell culture plastic dishes at a density of $2\times10^4$ cells/$cm^2$ [15], referred to as standard cell density (SCD). Low cell density (LCD) cultures were seeded at 10 cells/$cm^2$. MTX treatment was always initiated 18–24 h after seeding using 1 ml of Dulbecco's Modified Eagle Medium (DMEM) plus 10% of fetal bovine serum (FBS) per square centimeter of culture plate, and the medium was changed every 2 days. DMEM was fully supplemented to allow complete presence of metabolite sources. Cell cultures were maintained in humidity-saturated incubators at 37 °C in an atmosphere of 7.5% $CO_2$. Cell growth was measured by direct cell counting on Neubauer chambers of cell suspensions obtained by trypsin treatment.

HT-29 cell populations stably resistant to 0.1 and 1 μM MTX, designated as HT-29 M7 and HT-29 M6, respectively, were obtained as described previously [15]. Briefly, to obtain the HT-29 M7 population, parental HT-29 SCD

cultures were continuously treated with 0.1 μM MTX for 1 month and thereafter passaged weekly at the same cell density in the presence of 0.1 μM MTX for 3 months. The HT-29 M6 cell population was obtained by the same protocol except that an HT-29 M7 population was treated with 1 μM MTX [15].

### 2.2. Conditioned media and clonogenic assays

Conditioned media were routinely obtained as supernatants of 2-day-treated cultures or control untreated cultures; medium collection was initiated the third day after seeding. Conditioned media were filtered with 0.2-μm polycarbonate devices (Millipore) and stored frozen until used. For clonogenic assays, LCD cultures were treated the day after seeding for the indicated periods of time with conditioned media or with fresh medium pre-incubated at 37 °C for 2 days. Colony counting was performed with the naked eye on Coomassie blue-stained plates. Duplicates for each experiment were counted, and data shown was representative of at least two independent experiments.

### 2.3. Kinetics of intracellular thymidine transport

Thymidine transport kinetics was measured as previously described [19]. After the treatments indicated in the text, cells cultured in 24-well plates were rinsed three times in HEPES-buffered Ringer's solution (135 mM NaCl, 5 mM KCl, 3.33 mM $NaH_2PO_4$, 0.83 mM $Na_2HPO_4$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES; pH 7, 4) and preincubated in the same buffer at room temperature for 10 min. HEPES-buffered Ringer's solution containing increasing concentrations of [methyl-$^3$H]thymidine (39-46 Ci/mmol) (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) supplemented with an excess of cold thymidine (1:1000) was then added to the wells, and thymidine was allowed to be incorporated for 4 min at room temperature. Linearity of thymidine uptake during a total 8-min incubation time was confirmed at the lowest and highest concentrations used. After incubation, cells were washed three times in ice-cold PBS (pH 7.4) and lysed in 1% SDS, 1 mM sodium orthovanadate, 10 mM Tris–HCl (pH 7.4). Radioactivity in the cell samples was measured using a β-scintillation counter.

### 2.4. Nucleoside culture medium depletion assays

[Methyl-$^3$H]thymidine or [8-$^3$H]hypoxanthine (28 Ci/mmol) was added, at 1 nM, to the culture medium of cells seeded on 24-well plates and treated as indicated in the text. Samples of the culture medium were taken at different time points after incubation, and nucleoside depletion was calculated as the percentage of radioactivity remaining in the medium with respect to labeled medium incubated with no cells.

## 3. Results

### 3.1. HT-29 colon cancer cell resistance to acute MTX treatment is due to a transient adaptive mechanism

To study the cell population dynamics during MTX treatment, we first analyzed the kinetics of cell death and cell survival during continuous treatment. As previously described [15], treatment of HT-29 cells with 0.1 μM MTX abrogated cell proliferation and led to massive cell detachment starting on day 4 of treatment (Fig. 1A). Loss of cell viability was first detected at day 1 of treatment (Fig. 1B), thus preceding cell detachment. After a nadir of viability at days 2–3 of treatment, a transient recovery was observed which appeared to originate from no more than 1% of the initially seeded cells (Fig. 1B). This recovery phase allowed

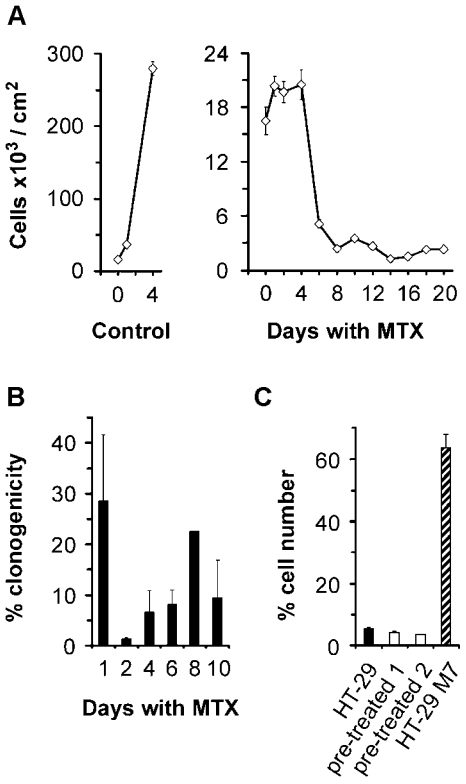


Fig. 1. MTX induces HT-29 cell death, and the surviving cell population displays a self-limited growth capacity in the presence of MTX. (A) HT-29 cells were seeded at $2\times10^4$ cells/cm$^2$, and 0.1 μM MTX treatment was initiated 24 h later; cell density kinetics of untreated (left) and MTX-treated (right) cultures are shown (ordinates, ×1000 cells/cm$^2$). (B) Changes in the clonogenic capacity of treated cells relative to untreated control cells shown in A. Cell samples were taken from the cell population remaining attached after the indicated time periods of treatment and cultured at clonogenic cell densities in control medium for 10 days. The number of colonies formed was counted by the naked eye. (C) Early resistance of HT-29 cells to MTX is due to reversible adaptation. Cell growth relative to untreated controls after 5 days of 0.1 μM treatment of two cell populations surviving a previous 10-day course of MTX treatment (pretreated 1 and pretreated 2), a stably resistant HT-29 cell population obtained by chronic treatment with 0.1 μM MTX (HT-29 M7) and previously untreated control cells (HT-29). Mean values of duplicates±mean error of representative experiments are shown in A and B, and of two independent experiments in C.

the proliferation of surviving cells in the form of growing colonies during continuous treatment, indicating that survival to MTX occurred in actively proliferating cells. However, this surviving cell population could not reach cell density values higher than 2–4×10$^3$/cm$^2$ at any time during the course of treatment (Fig. 1A), even after several weeks, which is a culture condition that we have named as "critical cell density" (see below). At this density, cells were resistant up to 100 μM, a MTX concentration that is 2000-fold higher than the ID50 of HT-29 cells (0.05 μM).

One possibility that could explain the survival of HT-29 cells to MTX is that a minor subpopulation of cells intrinsically resistant was selected during acute treatment (inherent resistance). To examine this possibility, cell populations surviving a 10-day course of 0.1 μM MTX were isolated, expanded in the absence of the drug, and thereafter treated again with MTX. If an intrinsically resistant cell subpopulation indeed existed, cells surviving the first course of MTX treatment (i.e., less than 1%; see Fig. 1B) would readily outgrow upon treatment with a second course of MTX. By contrast, pretreated cells were found to be as sensitive to MTX as untreated HT-29 cells (Fig. 1C). This behaviour is unlike that of cell populations that had been chronically cultured in the presence of 0.1 μM MTX (HT-29 M7 population), which possess stable resistance (Fig. 1C; Ref. [15]). Therefore, HT-29 cell survival to acute MTX treatment is due to an adaptive mechanism of reversible resistance rather than selection of inherent or acquired resistance.

### *3.2. HT-29 cell sensitivity to MTX is dependent on the cell culture density*

The results described above showed an association between cell density and MTX sensitivity and suggested a causal relationship between them. To assess this possibility, we seeded HT-29 cells within a range of cell densities, treated them for 5 days with 0.1 μM MTX beginning the day after seeding, and analyzed their response to the drug in terms of cell growth and cell viability. As shown in Fig. 2A, cells seeded at extreme low densities (10 to 300 cells/cm$^2$) displayed a clonogenic capacity similar to that of untreated cultures although they showed a notable growth decrease. At higher seeding cell densities (between 300 and 1000 cells/cm$^2$), both the clonogenic capacity and cell growth were dramatically reduced in comparison to untreated controls (Fig. 2A). Therefore, MTX-induced cytotoxicity is strictly dependent on cell culture density, low cell density (LCD) cultures being resistant to MTX.

Since 10-day pretreatment with MTX did not reveal inherent drug resistance (Fig. 1C), we next analyzed whether the surviving colonies from LCD-treated cultures displayed stable versus reversible resistance. Cultures initially seeded at 10 cells/cm$^2$ and treated with 0.1 μM MTX showed exponential growth until day 8 of treatment (Fig. 2B). At this point, cells became sensitive to MTX: (i) the total number

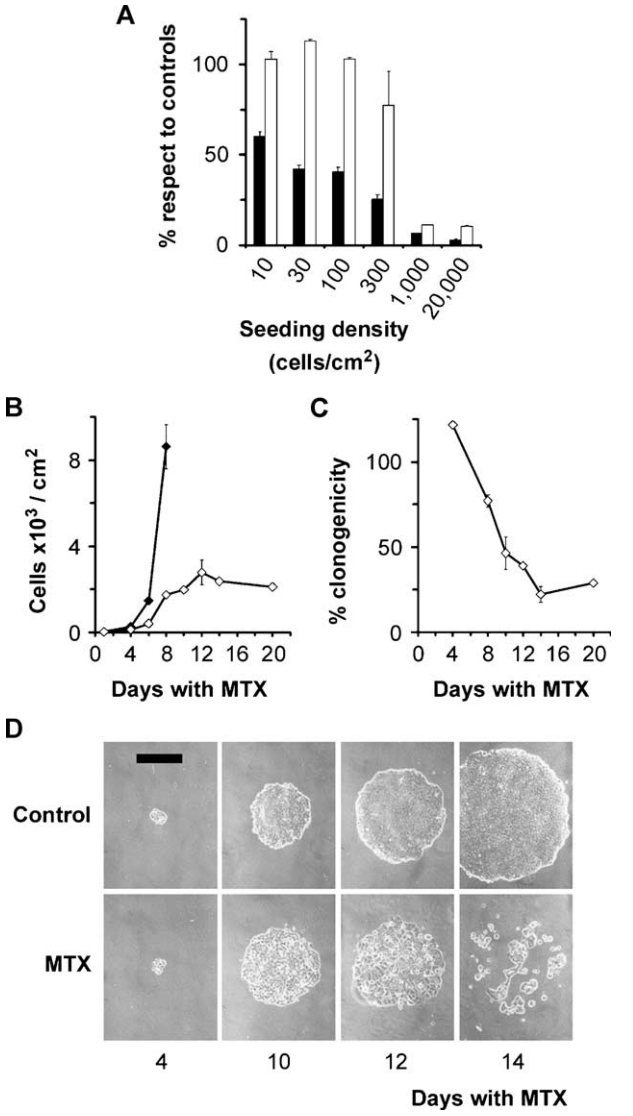


Fig. 2. A "critical cell density" defines growth conditions below which HT-29 cells are resistant to MTX. (A) Cell growth (solid black) and clonogenicity (solid white) after 5 days of treatment with 0.1 μM MTX at the range of seeding cell densities indicated; results are expressed as percentage relative to untreated cultures. (B) Cell density kinetics (ordinates, ×1000 cells/cm$^2$) of cells seeded at LCD during a course treatment with 0.1 μM MTX (white diamonds) with respect to untreated controls (black diamonds). (C) Clonogenicity of cultures treated as in panel B relative to that of untreated cultures; cell samples were taken at the indicated times and clonogenicity assays performed as described in Fig. 1B. (D) Phase-contrast microscopy of representative untreated and 0.1 μM MTX-treated colonies at the indicated times of treatment from the experiment shown in B. Bar, 250 μm. Mean values of duplicates±mean error of representative experiments are shown in A–C.

of cells/plate reached a plateau, which coincided with the "critical cell density" described in Fig. 1A (Fig. 2B); (ii) a gradual loss of clonogenic capacity was observed (Fig. 2C); and (iii) colonies showed cell detachment and fragmentation (Fig. 2D). These results thus demonstrate that exposure of HT-29 cells to MTX at densities below the "critical cell density" permits cell survival and exponential growth; when the "critical cell density" is reached, cells become sensitive to

MTX and cell death ensues. Again, cell survival to MTX is shown to occur in actively proliferating cells.

### 3.3. HT-29 cells adapt to MTX treatment by limiting the cell culture density attained

We hypothesized that the cell density-dependent sensitivity to MTX might be due to changes in the composition of the culture medium. To test this hypothesis, we performed cell clonogenicity assays in two-chamber transwells seeded at different cell densities. The results showed that there was a significant suppression of clonogenicity in a LCD-seeded culture when it was exposed to an SCD culture in the presence of MTX (Fig. 3A). By contrast, exposure to either another LCD culture or to plain medium had no effects on colony formation (Fig. 3A). A similar effect could be demonstrated when treating LCD-seeded cultures with 2-day conditioned medium from MTX-treated SCD cultures but not from LCD cultures (Fig. 3B). The effect of SCD-conditioned medium could only be evidenced when the clonogenic assay was carried out in the presence of MTX (Fig. 3A and 3B). Altogether, these experiments show that sensitization of HT-29 cells to MTX occurs through a mechanism involving conditioning of the culture medium by the cell population, which so self-sensitizes in a cell density-dependent manner.

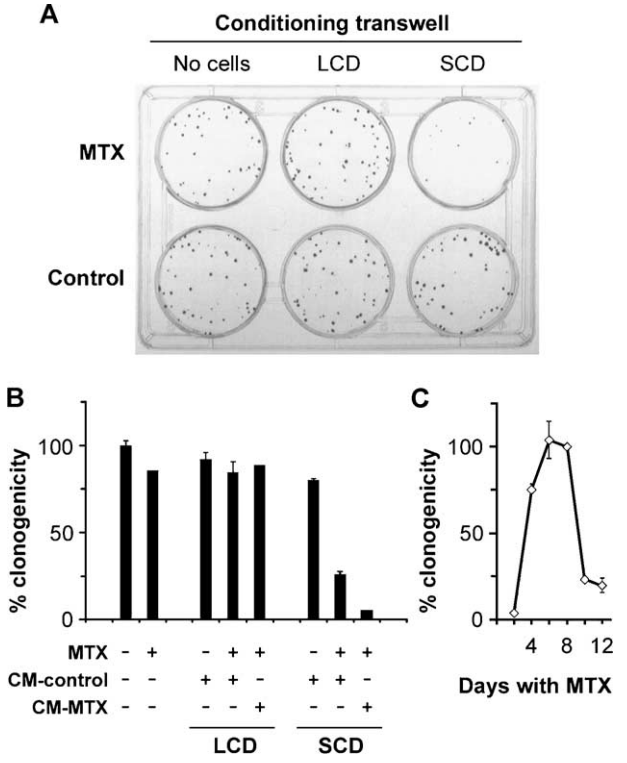


Fig. 3. Sensitization of HT-29 cells to MTX is due to conditioning of the culture medium at or above the "critical cell density", so that the cell population under treatment reduces its size to allow cell survival. (A) HT-29 cells were seeded at LCD on the bottom chamber of all wells, and cultures were exposed to a transwell chamber containing either no cells or HT-29 cells seeded at densities of 10 cells/cm$^2$ (LCD) or $2\times10^4$ cells/cm$^2$ (SCD) for 4 days. Cells were cultured (upper row) or not (lower row) in the presence of medium containing 0. 1 μM MTX. Clonal expansion of viable cells was allowed to proceed, after withdrawing the transwells, in control medium until day 10. (B) Clonogenic capacity of HT-29 cultures seeded at the LCD and treated for 3 days with 2-day conditioned medium from cultures seeded at either LCD (10 cells/cm$^2$) or SCD ($2\times10^4$ cells/cm$^2$) in the presence of 0.1 μM MTX (CM-MTX) or without the drug (CM-control). The presence of MTX at the time of the assay is indicated (MTX), whether included in the CM-MTX medium or added to CM-control medium. Control represents MTX-containing medium incubated for 2 days in the absence of cells; clonogenic capacity was evaluated after incubation in control medium until day 10. (C) Clonogenicity assays as in B testing conditioned media taken every 2 days from the treatments shown in Fig. 1A. Mean values of duplicates±mean error of representative experiments are shown in B and C.

We next analyzed the kinetics of conditioned medium production every 2 days during the course of MTX treatment in the SCD-seeded cultures shown in Fig. 1A. As expected, conditioning was high with the medium taken at day 2 of treatment, as in Fig. 3B (Fig. 3C). This conditioning capacity paralled the loss of cell viability observed in the original cultures (Fig. 1B). Interestingly, media collected between days 4 and 8 showed no capacity to suppress cell viability in the clonogenic assays (Fig. 3C), which is consistent with the recovery of cell viability observed in Fig. 1B, and suggests that this lack of conditioning activity allowed the survival and growth expansion of the 1% cell fraction surviving the first 2 days of treatment. Once this surviving cell population underwent growth expansion amid a majority of nonviable cells (Fig. 1B), the conditioning activity was again detected (Fig. 3C, days 10 and 12). Therefore, the cell density-dependent conditioning activity on the culture medium accounts for the periods of cell sensitization to MTX observed during continuous treatment. It can be concluded that, regardless of cell density at the beginning of the treatment, tumor cells adapt to a stable population size—i.e., the critical cell density.

### 3.4. HT-29 cells self-sensitize to MTX by depleting the extracellular nucleosides in the culture medium

One known mechanism to bypass the de novo pathway of deoxynucleotide synthesis inhibited by antimetabolites, particularly inhibition of thymidilate synthesis, is the supply of an extracellular source of nucleosides that will activate salvage nucleotide synthesis and replenish the intracellular pools of nucleotides [1]. Accordingly, we found that low density-dependent resistance was suppressed by MTX treatment combined with either of two chemically unrelated inhibitors of nucleoside membrane transport, DP and NBMPR. Moreover, exogenously added hypoxanthine plus thymidine rescued cell viability in LCD cultures treated with SCD-conditioned medium (Fig. 4A). Therefore, cell density-dependent resistance requires the salvage pathway of nucleotide synthesis, and the sensitizing effects of conditioned medium are due to inhibition of this pathway.

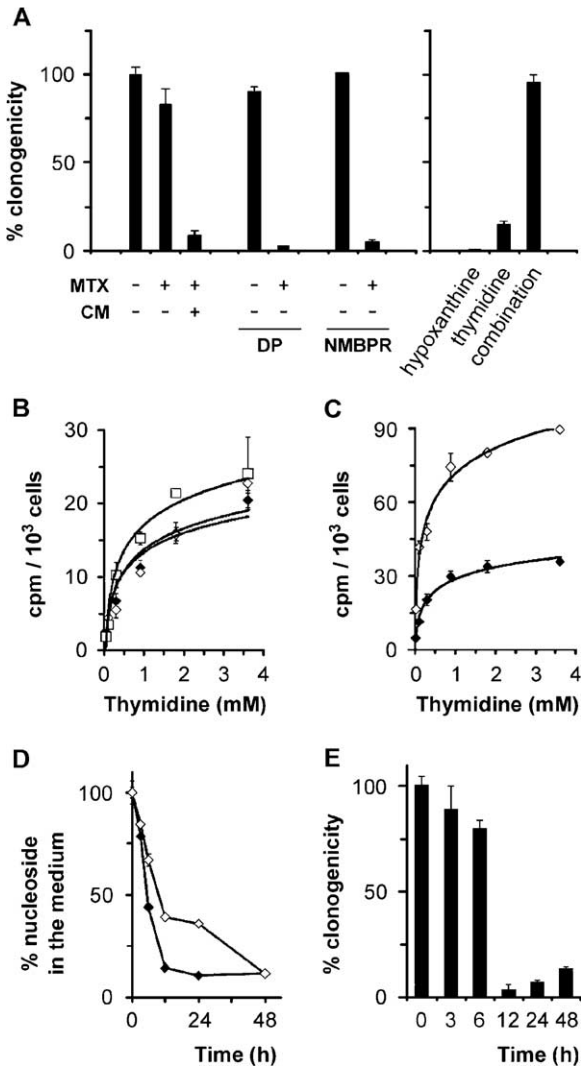


Fig. 4. Role of extracellular nucleosides in the low density-dependent resistance to MTX: resistance is dependent on extracellular nucleoside uptake, and the MTX-sensitizing effects of conditioned medium involve extracellular nucleoside depletion. (A) Clonogenicity assays, performed as described in Fig. 3B, testing the effect of the addition of DP (5 μM) or NBMPR (10 μM) on the resistance of HT-29 cells seeded at the LCD (left panel). In the right panel, the effect of adding thymidine (16 μM), hypoxanthine (100 μM), or a combination of both to medium conditioned by HT-29 cells seeded at the SCD and treated with 0.1 μM MTX (CM). (B) Intrinsic kinetics of $^{3}$H-thymidine cellular uptake by HT-29 cells seeded at the SCD untreated (black diamonds) or previously treated for 1 h with either 0.1 μM MTX (white diamonds) or medium conditioned by SCD-seeded, 0.1 μM MTX-treated cultures (white squares); uptake kinetic is expressed as intracellular cpm/1000 cultured cells (ordinates) with respect to the total thymidine concentration in the incubation buffer (abscissae) as described in Materials and methods. (C) As in B except that cells had been cultured in complete medium with (white diamonds) or without (black diamonds) 0.1 μM MTX for 12 h. (D) Time-course of $^{3}$H-thymidine (black diamonds) and $^{3}$H-hypoxanthine (white diamonds) depletion from the culture medium of HT-29 cells seeded at the SCD and analyzed the day after seeding. Results are shown as the percentage of radioactivity remaining in the culture medium (ordinates) at different time points (abscissae) during the incubation with MTX. (E) Clonogenic capacity of LCD cultures treated with conditioned media, as in Fig. 3B, obtained at different time points of the experiment shown in D. Mean values of duplicates±mean error of representative experiments are shown.

To assess whether inhibition of the salvage pathway occurred at the level of nucleoside cellular uptake (for example, due to the secretion of an inhibitory factor), we analyzed the intrinsic kinetics of radiolabeled thymidine intracellular accumulation by SCD-seeded cultures. We found that these kinetics were similar after the first hour of treatment when comparing control, MTX or conditioned medium (Fig. 4B). Moreover, the ability of 12-h-pretreated SCD cultures to accumulate radiolabeled thymidine (when their medium was already conditioned; see Fig. 4E) was not diminished but even increased with respect to nontreated controls (Fig. 4C). Hence, the capacity of conditioned medium to suppress cell density-dependent resistance is not due to a defective nucleoside cellular uptake machinery (including membrane transport and cellular retention by phosphorylation). Alternatively, we found that the sensitizing effects of SCD-conditioned medium could be reverted by a low molecular weight fraction present in the fetal bovine serum used in the culture medium (data not shown), suggesting a depletion of survival factor effect rather than active secretion of sensitizing factors. To ascertain whether extracellular nucleosides were the survival factors absent in the conditioned medium, we measured the capacity of SCD-seeded cultures to modify the extracellular concentration of nucleosides. Traces of radiolabeled thymidine or hypoxanthine were added to SCD cultures and the radioactivity remaining in the culture medium was measured during a time-course treatment with MTX. The results showed that most of the extracellular thymidine and hypoxanthine were depleted during the first 6 to 12 h of treatment (Fig. 4D), which is approximately the culture time period required to produce conditioned medium (Fig. 4E). Similar depletion kinetics were observed in cells cultured without MTX (data not shown). Folate concentration in conditioned medium was similar to that of control medium indicating that availability of folate is not limiting in this cell culture model (data not shown). These results altogether indicate that the capacity of conditioned medium to sensitize low density-dependent resistant cell cultures to MTX is due to depletion of extracellular nucleosides needed for salvage nucleotide synthesis.

### *3.5. Low density-dependent resistance to MTX is common in osteosarcoma and colon cancer cell lines*

We also analyzed other colon and non-epithelial cell lines with diverse oncogenic alterations: colon cancer SW480 and HCT116 [20], and osteosarcoma Saos-2 and U2-OS [21]. All the cells tested were sensitive to 0.1 μM MTX when seeded at SCD, that is $1–2\times10^{4}$ cells/cm$^{2}$ (data not shown), yet were resistant when seeded at LCD (10 cells/cm$^{2}$) (Fig. 5A). In addition, they all were able to condition the culture medium in the presence of MTX when cultured at SCD so as to suppress low density-dependent resistance either on their respective LCD cultures or on HT-29 LCD cultures (Fig. 5). Therefore, low density-dependent resistance occurs

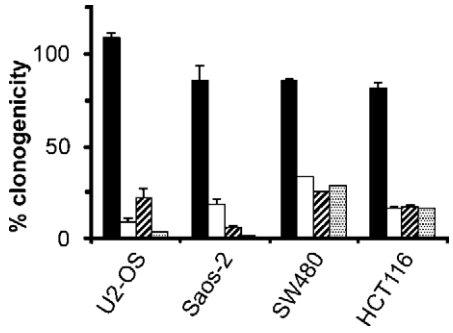


Fig. 5. Low density-dependent resistance is common in colon cancer and osteosarcoma cell lines. Clonogenic capacity of the indicated cell lines cultured in the presence of MTX alone (black solid), with conditioned media from their corresponding cultures seeded at the SCD and treated with 0.1 μM MTX for 2 days (white solid), or with similar conditioned medium from HT-29 SCD cultures (crosshatched bars). In dotted bars, clonogenic capacity of HT-29 cells using MTX-containing conditioned media from cultures of the indicated cell lines. Percentages in the ordinates are expressed relative to untreated controls. Mean values of duplicates±mean error of representative experiments are shown.

in different cancer cell lines, regardless of their origin or oncogenic alterations, and can be suppressed by a self-sensitizing mechanism occurring at SCD culture that is apparently similar to the one found in HT-29 cells.

## 4. Discussion

Our findings support the notion that tumor cells adapt to drug treatment by taking advantage of growth conditions that preserve the availability of these extracellular survival factors (i.e., maintenance of the cell population below a critical size): an SCD population self-sensitizes to MTX by depleting the extracellular nucleosides; by means of drug-induced cell death, the SCD population leads to an LCD population unable to condition the extracellular milieu, so that drug resistance becomes apparent. This behaviour reveals that not only the presence of survival factors, but the metabolic activity of tumor cells on the extracellular milieu is also determinant for the response to drug treatment. As opposed to selection of inherently resistant tumor cell subpopulations (Fig. 6), cell density-dependent adaptation is the first step of resistance in the present in vitro model in such a way that (i) it is dependent on the availability of extracellular nucleosides, which act as survival factors, and (ii) it precedes the eventual acquisition of permanent resistance as MTX treatment is allowed to proceed [15]. Indeed, our ongoing experiments indicate that sustained balanced cell death and cell survival at the critical cell density constitutes a setting of high cell turnover to allow the emergence of acquired resistance during chronic treatment (manuscript in preparation).

It has long been established that extracellular nucleosides can bypass the effects of antimetabolite drugs on the de novo synthesis of nucleotides [1,11–14]. For this reason, many studies seeking to investigate MTX action make use of culture media deficient in folate and/or nucleosides, and foetal serum used in culture is dialyzed to avoid metabolite supplementation. Our results show that using culture medium fully supplemented with untreated fetal serum indeed allows MTX cytotoxicity on tumor cells but only above a certain cell density culture. When cells are grown

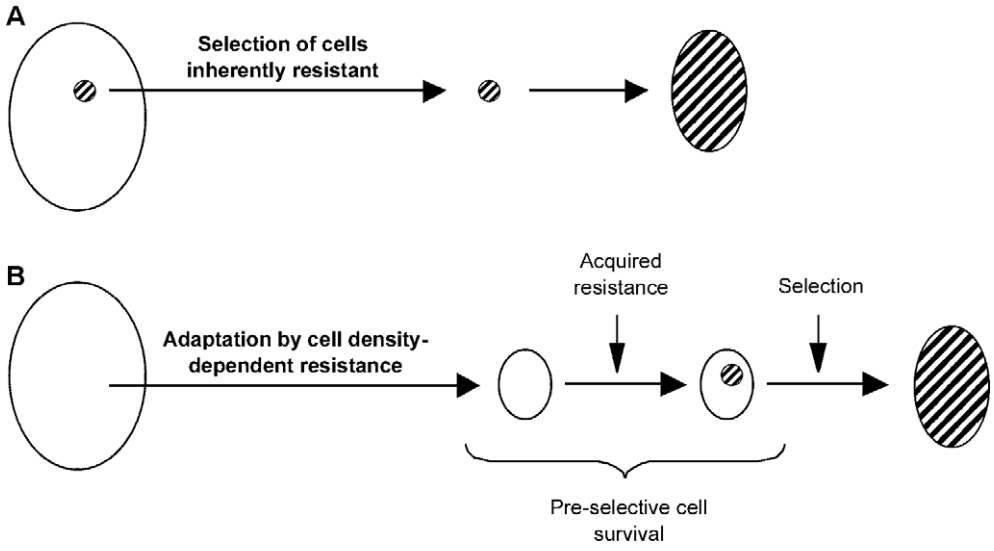


Fig. 6. Hypothetical model on two possible situations where tumors sensitive to an anticancer drug (e.g., MTX) escape from treatment to give rise to drug-resistant recurrent tumors. (A) Tumors may contain inherently resistant cell subpopulations (present before treatment and directly selectable by the drug) so that recurrences that are drug-resistant will emerge in spite of an initial tumor regression. (B) Tumors lacking inherent resistance may regress upon drug treatment to a size where the tumor microenvironment is compatible with the phenomenon of cell density-dependent resistance. In this situation, transiently resistant cell populations might survive and give rise to tumor recurrences. The situation described in panel A might only develop in B provided that mechanisms of permanent resistance are additionally acquired by the recurrent tumor; in such a case, cell density-dependent resistance appears as a preselective cell survival mechanism because the resistant subpopulation will be selected by cell density-mediated pressure. Solid white circles represent the drug sensitive tumor population and crosshatched circles represent cell subpopulations with inherent resistance.

above this cell density threshold, extracellular nucleosides are rapidly depleted (i.e., 12 h) and cells self-sensitize to MTX. Such phenomenon defines a "critical cell density" which appears as a culture situation of sustained cell survival during continuous treatment (see Fig. 1A) due to a counterbalance between culture medium refreshment and cell-mediated depletion of extracellular nucleosides.

We have named this type of reversible resistance to MTX as "cell density-dependent adaptation". Noteworthy, the "critical cell density" was not a fixed parameter as it could be raised by increasing either the amount of culture medium or the frequency of culture medium change (data not shown), indicating that the actual parameter determinant for MTX sensitivity is the amount of new culture medium available per cell for a given time period. It is also important to note that cell density-dependent adaptation does not involve changes in the growth state of the cell population under treatment: LCD cultures displaying resistance divide exponentially and self-sensitize at the very exponential phase of colony growth, as soon as they reach the critical cell density (Fig. 2B).

Although still speculative until in vivo studies confirm the clinical relevance of this mechanism, the cell density-dependent resistance provides a putative explanation for the behaviour of some tumors refractory to antimetabolite-based therapies, i.e., tumors outgrowing after initial shrinkage or from minimal residual disease. The concentration of serum nucleosides fluctuates during MTX treatment in both rats [22] and humans [23], suggesting that mechanisms of extracellular conditioning similar to those described in the in vitro model also occur in vivo. Accordingly, we have estimated that the concentrations of thymidine and hypoxanthine in combination to suppress in vitro 50% of low density-dependent resistance are 0.16 and 0.10 μM, respectively, which are values that range the concentrations found in serum [22–24]. Moreover, areas of the tumor mass associated with poor angiogenesis are restricted in nutrient supply, such as glucose, and may possibly be also limited in the availability of extracellular nucleosides. It is then possible that nucleosides, as well as other survival factors, are depleted by tumor cells in these areas more easily than in small tumors or micrometastasis, a possibility that would then implicate tumor size as a determinant of MTX sensitivity. In this regard, little is known about the metabolic conditions in areas of necrosis that may modulate response to chemotherapy. It has also been suggested that necrotic cells release nucleosides and other molecules to the extracellular milieu that can rescue surviving cells from MTX-induced cytotoxicity [25,26]. This issue may be particularly important in tumors, such as colorectal cancers, characterized by poor angiogenic response and growth with extensive areas of central necrosis. Therefore, differential depletion of extracellular nucleosides and other extracellular survival factors by tumor cell populations of diverse size provides a putative explanation to why certain types of tumors—such as colorectal carcinoma—appear sensitive to MTX but cannot be led into complete remission.

We ruled out changes in the kinetics of nucleoside cellular uptake as responsible for the cell density-dependent sensitivity to MTX in vitro, but modulation of transport activity—in addition to substrate availability—might also be an important determinant of cell sensitivity to MTX in vivo. Accordingly, several nucleoside transporters have been cloned in the last years [27,28], and their expression can be modulated by either nucleoside availability or antimetabolite treatment, both in vitro and in vivo [29–32]. We also found increased thymidine intracellular accumulation as a consequence of medium conditioning during MTX treatment (Fig. 4), which might be due to increased transport or to increased phosphorylation by thymidine kinase in order to replenish the intracellular pools of thymidilate. Finally, these transporters may also play a role in the acquisition of antimetabolite drug resistance, in addition to inherent resistance, as equilibrative nucleoside transporter 1 is among the genes overexpressed in gastric cancer cells resistant to 5-fluorouracil [33].

In summary, the cell density-dependent model illustrates a process of tumor cell adaptation to MTX treatment at the early stages of drug resistance, thus linking the known action of extracellular metabolites as survival factors to drug treatment with a novel insight into the dynamic interaction between tumor cells and their microenvironment.

## Acknowledgements

The authors thank Coral Ampurdanés for technical assistance; Clara Mayo, Pilar García-Morales, Ángeles Gómez-Martínez, and Alfredo Carrato for collaborative support; Carlos Ciudad and Marçal Pastor-Anglada for helpful comments to a prior version of the manuscript. This work was supported in part by grants from Ministerio de Ciencia y Tecnología (FEDER 1FD97-0902, SAF2001-0434), Fondo de Investigaciones Sanitarias (FIS01/1337), and a contract grant from Aventis. X.M. is supported by a contract from the Instituto de Salud Carlos III.

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