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Methotrexate resistance *in vitro* is achieved by a dynamic selection process of tumor cell variants emerging during treatment

Josep M. de Anta[1], Clara Mayo[1], Francesc Solé[2], Marta Salido[2], Blanca Espinet[2], Cristina Corzo[2], Myriam Petzold[1], Olaya Villa[2], Sergi Serrano[2], Francisco X. Real[1,3] and Xavier Mayol[1]*

[1]*Unitat de Biologia Cel·lular i Molecular, Institut Municipal d'Investigació Mèdica, Barcelona, Spain*
[2]*Laboratori de Citogenetica i Biologia Molecular, Servei de Patologia, Hospital del Mar, Unitat de Recerca Traslacional en Tumors Sòlids – PRBB, Barcelona, Spain*
[3]*Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain*

Genetic instability leads to tumor heterogeneity, which in turn provides a source of cell variants responsible for drug resistance. However, the source of resistant cells during the process of acquired resistance is poorly understood. Our aim has been to characterize the mechanism by which acquired resistance to methotrexate emerges during the course of cancer cell treatment in vitro. We recently demonstrated that, *in vitro*, HT-29 colon cancer cells become transiently sensitive to methotrexate by depleting the extracellular milieu of survival factors; on the other hand, the cell population under treatment can reversibly adapt to grow below a critical cell density in the presence of the drug. Here, we show that this adapted cell population gives rise to permanent resistant populations through repeated cycles of cell death and growth. This increased cell turnover, but not merely cell proliferation, is required for the appearance of increasing degrees of stable resistance that are progressively selected by drug pressure. Such a process, taking place in multiple steps, is here designated "dynamic selection." The analysis of sensitive and resistant HT-29 cell populations revealed that methotrexate induces genomic instability—characterized by centrosome amplification and aberrant chromosome recombination—leading to a low-level amplification of the 5q chromosome arm as one of the earliest genetic events selected during treatment. Therefore, this model provides a mechanism by which a tumor cell population lacking resistant subpopulations before treatment is able to acquire the genetic changes required for stable drug resistance.
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The genetic instability of cancer cells leads to chromosomal gains, losses or rearrangements, and/or elevated rates of gene mutation that play a major role in tumor progression.[1] Such instability leads to genetic heterogeneity required for the emergence of increasingly aggressive tumor cell populations, which undergo selective pressure following a darwinian process.[2] One instance of selective pressure impinging on tumor progression is drug treatment, so that tumor cell variants with increased drug resistance, e.g., less prone to apoptosis, can be selected during treatment. However, the processes that drive the appearance of drug-resistant tumor cell subpopulations as a result of treatment are not clear. In this respect, acquired resistance deviates from the darwinian notion of tumor progression in that it appears as an adaptive genetic or epigenetic response to drug treatment, analogous to the adaptive mutation observed in bacteria.[3]

Increased cell survival upon drug treatment has been proposed to occur in certain tumor microenvironments, such as in the bone marrow in the case of some hematopoietic malignancies.[4] In such instances, a combination of cell adhesion and soluble molecules may confer, following drug exposure, a survival advantage to some cells—the "survival niche"—that may facilitate the eventual emergence of acquired resistance.[5] Other situations, such as micrometastasis and/or hypoxia, might also require particular conditions for cell survival—a condition known as "tumor dormancy"—and in response to drugs.[6–10] In tumors that are uniformly drug-sensitive prior to drug exposure, adaptive cell survival would thus appear as an essential step for the eventual acquisition of stable resistance. We have recently reported that during methotrexate (MTX) treatment of tumor cells *in vitro*, cells overcome exposure to the drug because of the availability of survival factors that render them drug-resistant.[11] However, the population dynamics that link the adaptive cell survival step with acquisition of resistance are poorly understood. The identification of the adaptation, mutation and selection processes associated with drug resistance in tumors is thus of paramount importance.

We have recently shown that, *in vitro,* HT-29 colon cancer cells are killed by MTX when extracellular nucleosides, a salvage source of nucleotide precursors,[11,12] are depleted from the medium by the cell population under treatment. Thus, this effect depends on the relationship between the population size and the amount of nucleosides available. Accordingly, a "critical cell density" can be determined under which cells are resistant to MTX. Salvage nucleotide synthesis is known to be a mechanism of intrinsic resistance—as opposed to acquired resistance—in various tumor types, such as colorectal cancer.[12,13] On the other hand, treatment with MTX or other antimetabolites frequently leads to drug resistance, indicating that additional changes take place during treatment. In this respect, when HT-29 cells surviving to MTX by adaption to a low population size—and lacking inherently drug-resistant cell subpopulations[11]—are continuously treated with 0.1 μM MTX, permanent resistance arises.[14] This property can be demonstrated because it is still retained after drug withdrawal. While the number or nature of events responsible for the emergence of permanently resistant populations is uncertain, it has been shown that the stepwise selection of HT-29 populations by increasing the concentration of MTX (>0.1 mM) leads to high level amplification of dihydrofolate reductase (DHFR), the enzyme targeted by MTX.[15]

In this work, we analyze the cell population dynamics and mechanisms driving the emergence of resistance within a drug-sensitive tumor cell population, i.e. HT-29 cells treated with 0.1 μM MTX. We show that acquired resistance to MTX is, at least in part, genetic in nature and that it originates *de novo* during the cell density-dependent, transient resistance state by a combined process of increased cell turnover and sustained selective pressure. Only under this dynamic state of continued cell renewal can genetic instability give rise to variants with stable drug resistance. These variants appear stepwise with variable degrees of resistance found in particular cell clones. We therefore propose an *in vitro* model that dynamically selects for drug resistance and that may

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Josep M. de Anta's current address is: Departament d'Anatomia i Embriologia Humana, Facultat de Medicina, IDIBELL, Campus de Bellvitge, Universitat de Barcelona, Barcelona, Spain.
*Correspondence to: Unitat de Biologia Cel lular i Molecular, Institut Municipal d'Investigació Mèdica, C/Dr. Aiguader, 80, 08003 Barcelona, Spain. Fax: +34-93-221-32-37. E-mail: xmayol@imim.es
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allow to dissect the complexity of cell population dynamics during tumor progression.

Material and methods

Cells and cell culture

HT-29 cells were obtained from ATCC and used at passages 180–200. The HT-29 M7 populations were obtained by treatment of HT-29 cells with 0.1 μM methotrexate (MTX) during 30 days and thereafter passaged weekly for 3 months in the continuous presence of 0.1 μM MTX; after drug withdrawal, HT-29 M7 cells were used at passages between 5 and 15. HT-29 cells resistant to 1 μM (10^{-6} M) MTX, designated as the M6 population, were kindly donated by Thécla Lesuffleur and Alain Zweibaum (INSERM, France), and were obtained by subjecting an HT-29 M7 cell population to treatment with 1 μM MTX for 30 days.[14] Cultures were passaged by trypsinization every 2–3 days using Dulbecco's Modified Eagle Medium with 10% of fetal bovine serum. All the experiments were performed in humidified incubators at 37°C in an atmosphere of 7.5% CO_2.

Drug treatment

MTX was obtained as a 2.2 M solution (Almirall, Barcelona, Spain); stocks were prepared in culture medium at 10 mM and stored frozen until used. Dipyridamole (DP) was purchased from Sigma (St. Louis, MI). MTX treatment was always initiated 18–24 hr after seeding using 0.1 ml of medium per cm^2 of culture surface, and the medium was changed every 2 days. Culture media contained standard supplements of folate and amino acids to provide metabolites required for salvage nucleotide synthesis during cell density-dependent resistance culture situations.

Individual colony populations were obtained by treating HT-29 cell cultures, seeded at 2×10^3 cells/cm^2, with 0.1 μM MTX for 60 days. Surviving colonies without signs of fragmentation were individually isolated by aspiration with a micropipette tip and expanded in the absence of MTX. These clones were used at passages 5–15 after drug withdrawal; no differences in MTX sensitivity were noted within this passage range (data not shown).

Conventional cytogenetic analysis

The various HT-29 cell populations were subjected to G-banding karyotype analysis according to conventional procedures. Briefly, exponentially growing cell cultures without MTX were treated with colcemid for 1 hr; cells arrested in mitosis were harvested by gentle scraping and incubated for 15 min at 37°C in hypotonic buffer (KCl 0.075 M), fixed with methanol:acetic acid (3:1), washed 3 times with the same fixative, and used immediately or stored at −20°C in the fixative solution. Chromosome extension was performed on histological glass slides by dropping a small amount of the fixed cell suspension, and chromosomes were stained with Wright's solution.

FISH analysis

MTX-treated populations were hybridized with a 125 kb bacterial artificial chromosome probe containing dihydrofolate reductase (DHFR) genomic DNA, which was kindly donated by J.L. Hamlin.[16] DNA was isolated using standard alkaline lysis techniques (Qiagen). One microgram of each cloned DNA was labeled with SpectrumGreen-dUTP by nick translation (Vysis). Metaphase spreads from cell lines were pretreated with a solution of pepsin for 10 min at 37°C to digest cytoplasmatic membrane proteins. After a 5-min denaturation in 70% formamide at 74°C, the probe was hybridized to slides for 17 hr in a humidity chamber. Slides were then washed 3 times in 50% formamide, 2XSSC and 0.1% NP40 at 45°C. The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI II). Results were visualized using an Olympus BX51 fluorescence microscope, and images were captured using Cytovision software. DHFR signals were counted in a total of 200 interphasic nuclei for each HT-29 cell population.

Immunofluorescence

Cells growing on coverslips were fixed with 2% paraformaldehyde in PBS for 15 min at 4°C and stored in PBS. To detect mitotic spindles, immunofluorescence was performed using a monoclonal antibody against α-tubulin, clone DM 1A (Sigma; St. Louis, MI), followed by an FITC-conjugated anti-mouse Ig.

Western blotting

Cells cultured as described above were harvested, washed with PBS, and protein extracts obtained using lysis buffer containing Tris-HCl 0.1 M pH 7.2 and 0.1% SDS. Ten micrograms of total protein was loaded onto 10% acrylamide gels and subjected to electrophoresis under denaturing conditions. Proteins were electrotransferred to nitrocellulose membranes and immunoblots performed using anti-DHFR monoclonal antibody clone 49 (BD Biosciences) and horseradish peroxidase-conjugated goat anti-mouse (Dako) as a secondary antibody. DHFR signal was revealed by using an enzyme chemoluminiscence reagent (Amersham).

Results

The switch from adaptive resistance to permanent resistance is associated with extracellular nucleoside-independent growth

The HT-29 line does not contain cell subpopulations inherently resistant to MTX. Rather, when permanently treated with 0.1 μM MTX, HT-29 cultures maintain a cell density, designated "critical cell density," that is sufficiently small to permit extracellular survival factor-dependent resistance ("cell density-dependent resistance").[11] These cells often display a flattened morphology. As expected, MTX-treated cells could reach the critical cell density either by proliferating, if seeded at a density lower than the critical one, or by cell death, if seeded at a density higher than the critical one (Figs. 1*a*–1*c*). After several weeks at the critical density, some large colonies emerged regardless of the prior treatment. These colonies could be distinguished by their larger size, lack of flattened morphology and increased cell compaction, sometimes containing dome-like structures or cell multilayers (Figs. 1*d* and 1*e*). The appearance of such colonies in the cultures was responsible for surpassing the critical cell density. Conceivably, survival factor independence might involve a mechanism of resistance different from that involved in the "critical cell density" phenomenon.

To explore the basis of such mechanism, we randomly isolated large colonies from continuously-treated cultures. Individual colonies were expanded in the absence of MTX and each of these presumably clonal populations was seeded at 2×10^4 cells/cm^2 and treated for 5 days with 0.1 μM MTX. At this concentration, parental cells display massive death. In comparison with parental HT-29 cells, as well as clonal populations obtained from them by limiting dilution, one third of the isolated colonies displayed increased resistance to MTX and reached a population size of up to 70% with respect to nontreated controls (Fig. 2*a*). This growth capacity in the presence of MTX was similar to that of 2 independent HT-29 M7 populations stably adapted to MTX. Therefore, some colonies showed acquisition of "permanent" MTX resistance, since the continued presence of MTX was not required. We have previously shown that HT-29 line does not contain any MTX-resistant cell subpopulation prior to treatment with this drug,[11] indicating that the "critical cell density" growth phenomenon is a transient adaptation that can lead to permanent resistance.

We next assessed whether permanent resistance involved independence from extracellular nucleosides. The number of viable HT-29 colonies after 10 days of MTX treatment was reduced nearly to 0 by the nucleoside membrane transport inhibitor DP used in combination with 0.1 μM MTX for 16 additional days. By contrast, an increasing number of MTX + DP-resistant colonies

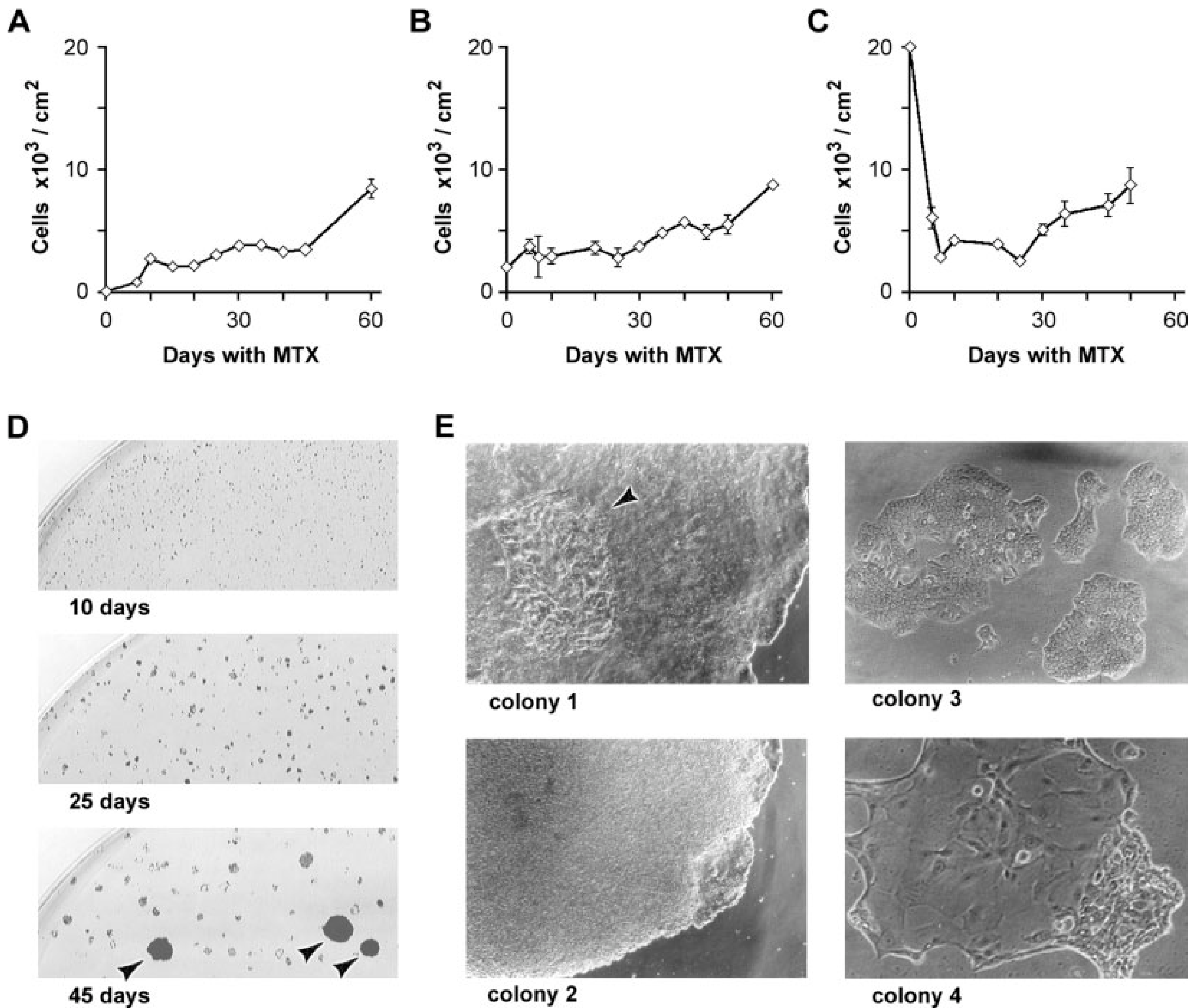

FIGURE 1 – Resistance features additional to cell density-dependent resistance to MTX are acquired after a period of transient resistance under continuous drug treatment. HT-29 cells were seeded at 3 different culture densities, (*a*) 10 cells/cm^2, (*b*) 2×10^3 cells/cm^2, (*c*) 2×10^4 cells/cm^2, treated with 0.1 μM MTX from the following day, and samples were taken at the indicated days for cell counting. The cell survival period that limits the cell population to a critical cell density (between 2 and 4×10^3 cells/cm^2) varied slightly with the initial seeding density before some large colonies emerged with unrestrained growth (arrowheads in (*d*)). These colonies were responsible for the increase in cell numbers observed at the latest time points in (*a*)–(*c*). (*e*) Details of unrestrained growing colonies displaying either differentiated features (colony 1; arrowhead pointing to a dome-like structure) or a highly packed multilayer form (colony 2), in contrast to the flattened and/or fragmented appearance of most of the colonies undergoing sensitization (colonies 3 and 4); note the heterogeneous phenotype in colony 4, which is representative of some colonies during the treatment.

emerged as the single MTX treatment was extended to 1 and 2 months (Fig. 2*b*). After 3 months of MTX treatment, nearly every colony was resistant to MTX + DP. These results indicate that within the cell population adapted to growth in MTX, which is critically dependent on extracellular nucleosides, some cells acquire permanent resistance and at the same time become independent from extracellular nucleosides for survival.

Dipyridamole allows to distinguish different degrees of permanent resistance

To characterize with more detail the acquisition of extracellular nucleoside independence, we analyzed resistance to a 5-day course of treatment with either MTX or MTX + DP in each of the cell populations shown in Figure 2*a* using 2 parameters: cell survival, measured as clonogenic potential, and cell proliferation. As shown in Figure 2*c*, there was a logarithmic relationship between clonogenicity and cell growth in the presence of MTX alone for each of the isolated populations: some of them displayed little proliferation while they were highly clonogenic, already indicating a first level of MTX resistance. Figure 2*d* shows the relationship between clonogenic resistance to MTX and to MTX + DP: not all populations with increased resistance to MTX were able to survive upon treatment with MTX-DP, indicating that survival to MTX does not necessarily involve independence from extracellular nucleosides. Finally, Figure 2*e* shows the relationship between clonogenicity upon treatment with MTX + DP and proliferative capacity under treatment with MTX alone, revealing a linear correlation among populations showing diverse levels of resistance. Therefore, the process leading to acquired resistance to MTX

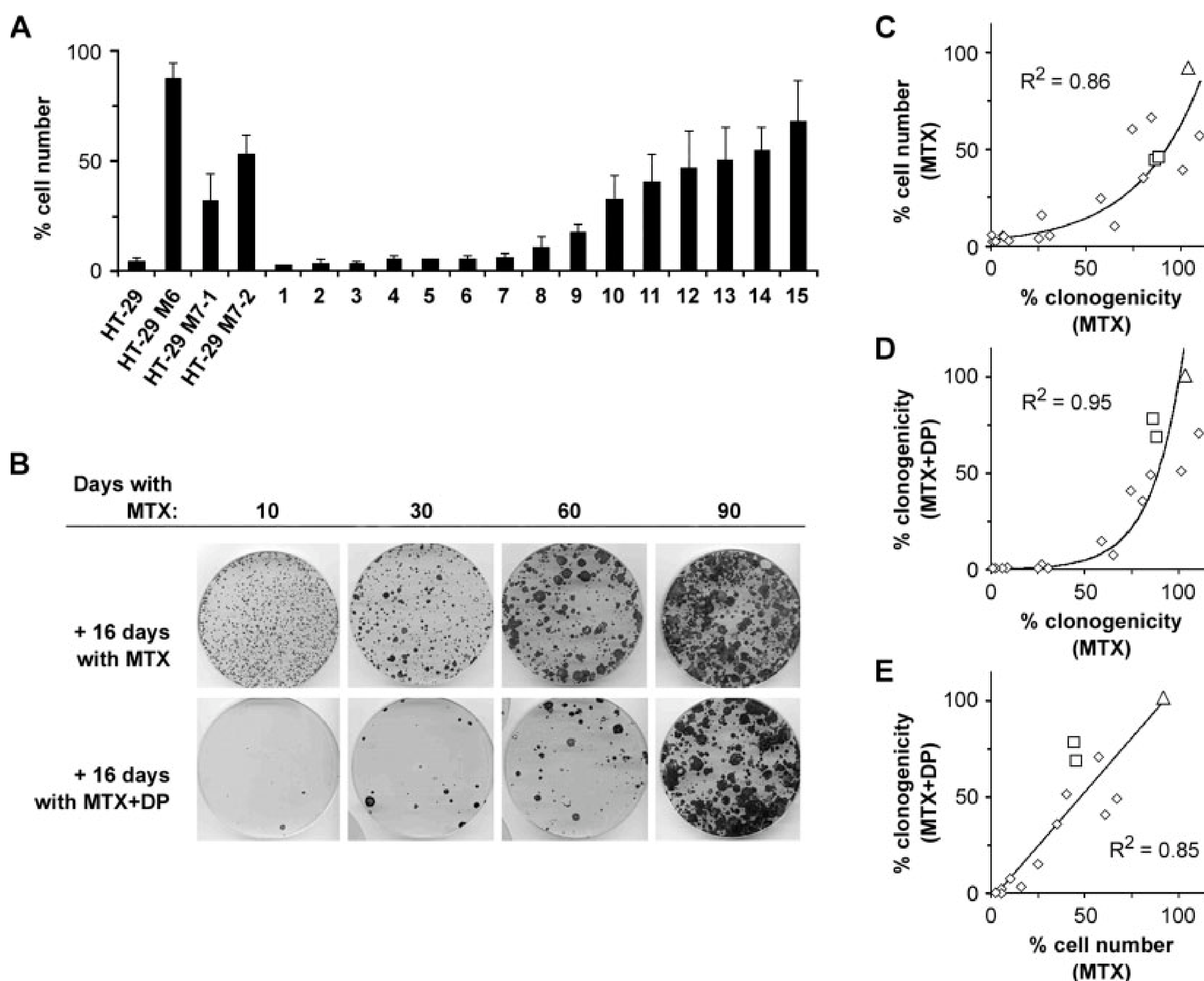

FIGURE 2 – Independence of salvage nucleotide synthesis during MTX treatment defines the acquisition of permanent resistance to MTX, which involves different degrees before attaining full resistance. (*a*) Permanent resistance was assessed as the capability of retaining resistance after withdrawing the drug and re-treating the resulting cell population at 2×10^4 cells/cm^2 with 0.1 μM MTX; only the cell populations derived from colonies 10–15 displayed a resistance to MTX comparable with that of 2 independently generated resistant populations obtained by chronic treatment with 0.1 μM MTX (HT-29 M7-1 and HT-29 M7-2), which have already been characterized as stably resistant.[14] HT-29, untreated cell population; HT-29 M6, resistant cell population isolated from an HT-29 M7 population that was additionally treated with 1 μM MTX. Results are shown as the number of cells obtained at day 5 of treatment in each population respect to its respective non-treated paired control. (*b*) The emergence of permanently resistant cell colonies amid the transiently resistant population occurs concomitantly with the acquisition of independence from extracellular nucleosides as survival factors to drug treatment; the proportion of cell colonies surviving to MTX that are insensitive to combined treatment with the nucleoside transport inhibitor dypiridamole increases with the length of MTX treatment indicating that permanent resistance is acquired progressively in some cell colonies. (*c*)–(*e*) HT-29 cells and clones 1 to 15 (diamonds), M7-1 and M7-2 populations (squares), and the HT-29 M6 cell population (triangles) were subjected to 5 days of treatment with 0.1 μM MTX alone (MTX) or 0.1 μM MTX and 1 μM DP (MTX + DP), and the percentages of cell growth (cell number) and cell survival (clonogenicity) were calculated relative to non-treated paired controls. (*c*) Some cell populations from those isolated in the experiment shown in (*a*) survive to MTX treatment (increased % clonogenicity (MTX)) but are unable to grow in the presence of MTX (low % cell number (MTX)). (*d*) As in (*c*), only some of the MTX-surviving clones are able to survive to MTX + DP combined treatment (% clonogenicity (MTX + DP)). (*e*) Full resistance appears to be achieved by increasing degrees of both growth capacity in the presence of MTX (% cell number (MTX)) and survival to MTX + DP combined treatment (% clonogenicity (MTX + DP)).

involves different degrees of permanent resistance suggesting that full resistance (i.e., capacity to survive as well as proliferate in the presence of the drug) is achieved in a multistep manner.

The process leading to acquired resistance consists of cycles of cell death and cell proliferation

To gain insight into the mechanisms responsible for the gradual emergence of resistant populations, we analyzed the growth kinetics of those populations that acquired permanent resistance. Cells were seeded at 2×10^4 cells/cm^2, and 80 colonies surviving after one week of MTX (0.1 μM) (see Fig. 1*a*) were randomly chosen and individually followed under phase contrast microscopy. Almost all colonies displayed a growth expansion phase from day 7 to day 10 of treatment (Fig. 3*a*), which was expected after the initial wave of cell death and before the critical cell density was reached.[11] Afterwards, several cycles of regression and expansion could be revealed when considering either all colonies

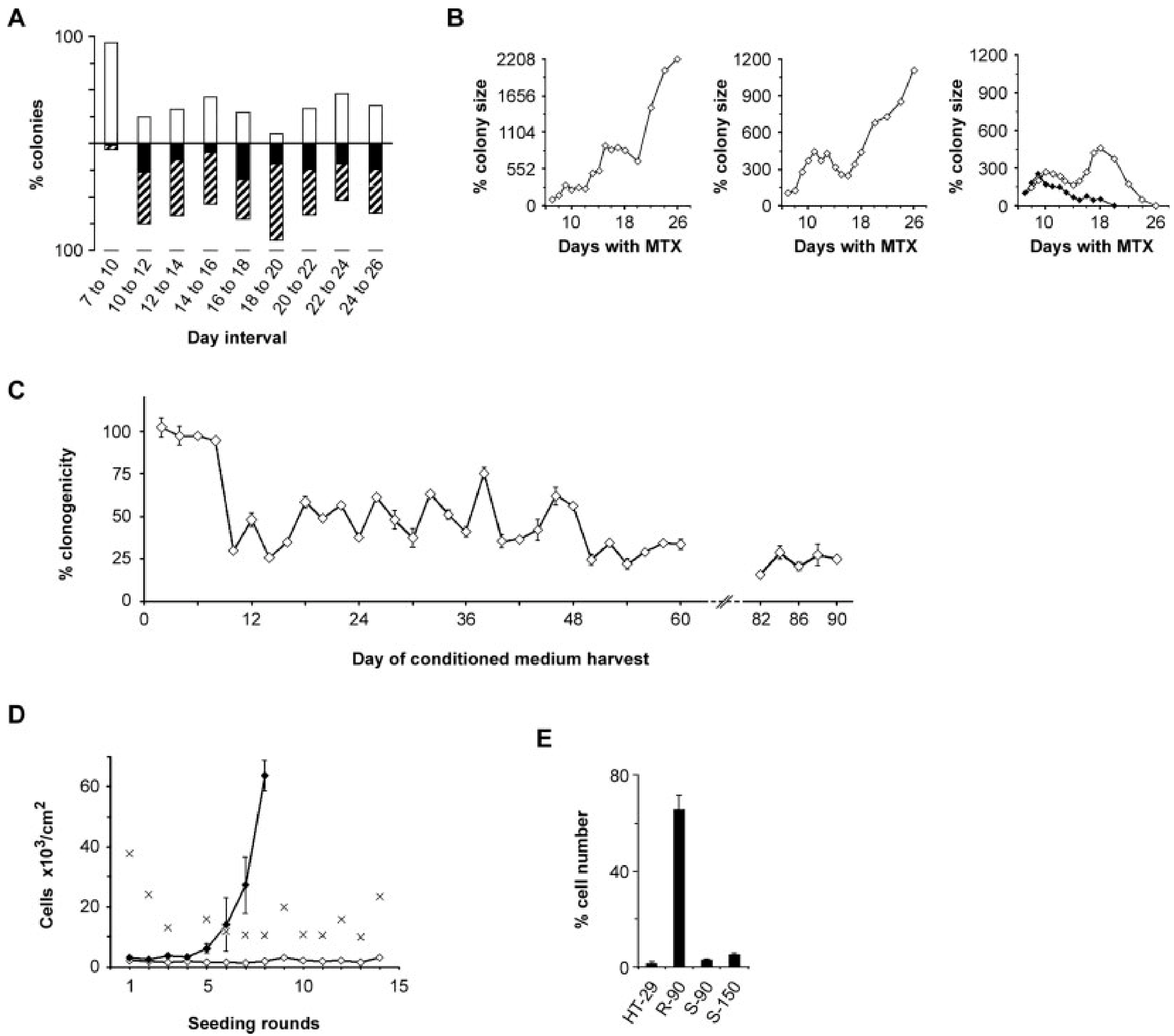

Figure 3 – Repeated cycles of cell death and growth recovery maintain the cell culture under treatment at a sustained critical cell density and are required for the acquisition of permanent resistance. (*a*) The kinetics of cell number variation during MTX treatment in 80 cell colonies—chosen from the experiment shown in Figure 1*b*—was calculated as the percentage of colonies increasing in cell number more than 10% (white bars), with variations of less than 10% (black bars), or decreasing more than 10% (cross-hatched bars) during the time period indicated in the ordinates respect to the size displayed in each preceding time period. (*b*) Representative examples of cell number variation from (*a*) measured as the percentual number of cells observed under the microscope in each colony at the indicated time points respect to the number of cells at the day seventh of treatment; two colonies perduring (left and center) and 2 colonies disappearing (right) are shown. (*c*) Conditioned media was collected every 2 days during the course of the treatment shown in Figure 1*a*, coinciding with medium changes, and clonogenic assays using untreated HT-29 cells were performed as described in Materials and Methods; clonogenicity was calculated as the percentage of colonies grown with each conditioned medium respect to clonogenicity with nonconditioned medium. (*d*) HT-29 cells were subjected to 2 series of 10-day rounds with 0.1 μM MTX treatment, each round initiated by seeding the cells obtained from the previous round either at 2×10^3 cells/cm^2 (black diamonds) or at 10 cells/cm^2 (white diamonds); results show the cell density attained in each round as the mean of duplicates ± standard error of a representative experiment; cross-lines indicate the cell number obtained by an additional 10-day round culture without drug of some populations of the 10 cells/cm^2 seeding protocol. (*e*) Permanent resistance is only attained in the multiround treatment protocol by seeding at 2×10^3 cells/cm^2; the untreated HT-29 cell population (HT-29), and the populations obtained from (*e*) using the seeding density 2×10^3 cells/cm^2 after the 9th round (R-9) or using the seeding density 10 cells/cm^2 after the 9th and 15th rounds (S-9 and S-15, respectively) were grown in the absence of MTX, subjected to a 5-day course treatment in the presence or absence of MTX at a sensitizing seeding density of 2×10^4 cells/cm^2, and the cells remaining at the 5th day counted; results are expressed as the percentage of cells remaining in MTX cultures respect to their respective nontreated cultures.

globally (Fig. 3*a*) or the majority of individual colonies (see examples in Fig. 3*b*). These cycles of death and proliferation led to the disappearance of ∼50% of the colonies in 4 weeks. The remaining colonies increased in size continuously over time.

As previously indicated based on our published work, the depletion of extracellular nucleosides is the critical event that renders HT-29 cells sensitive to MTX.[11] Therefore, one possibility that might explain these regression–expansion cycles is differential

conditioning of the culture medium in each cycle. To assess this hypothesis, we harvested conditioned media every 2 days during the treatment and analyzed their capacity to suppress the clonogenic growth of HT-29 cells seeded at very low density. The results showed that the clonogenic capacity fluctuated and was associated with the colony size variation shown in Figure 3*a*, at least during the first few cycles (Fig. 3*c*). Increasing the volume of fresh culture medium restored clonogenicity (data not shown). These results, therefore, indicate that the fluctuations in the availability of survival factors, among them extracellular nucleosides, are responsible for the regression–recovery cycles. Moreover, transient resistance involves a dynamic process, rather than a latency state, associated with the disappearance of some colonies and the persistence of others having gone through several cycles of cell loss and growth recovery, hence involving an elevated rate of cell turnover.

Increased cell turnover under continuous selective pressure is required for the acquisition of permanent resistance

Conceivably, increased cell turnover should favor both population heterogeneity and the selection of variants best adaptable to drug treatment. To determine its putative relevance in the process of acquisition of resistance to MTX, we designed 2 culture conditions that allow cell density-dependent resistance to MTX, but differ in the occurrence of the increased cell turnover: (*i*) cultures initially seeded at 2×10^3 cells/cm^2 and reseeded every 10 days at this density and (*ii*) cultures initially seeded at 10 cells/cm^2 and reseeded every 10 days at this density. In both cases, cultures were continuously treated with 0.1 μM MTX. Cultures seeded at 2×10^3 cells/cm^2 underwent cell proliferation and cell death from the beginning of each round, as expected, because cell density rapidly exceeded the critical cell density (Fig. 1*b*). In contrast, seeding at 10 cells/cm^2 allowed exponential growth until the tenth day, without any sign of cell death, as expected, because cell density is continuously below the critical cell density (Fig. 1*a*; Ref. 11). The results showed that the total cell number in cultures seeded at 2×10^3 cells/cm^2 rose above the critical cell density constraint after 6 reseeding rounds. These cultures progressively augmented their growth capacity in the presence of 0.1 μM MTX, indicating the progressive acquisition of resistance to MTX (Fig. 3*d*). In contrast, the cultures maintained using the 10 cells/cm^2 reseeding protocol displayed an inability to grow beyond the critical cell density, even after more than 15 seeding rounds, indicating that this constraint had not been overcome (Fig. 3*d*). To confirm that these differences in response to MTX were due to the acquisition of permanent resistance in the population undergoing the 2×10^3 cells/cm^2 reseeding protocol, cultures from these different conditions were expanded in the absence of the drug, seeded at 2×10^4 cells/cm^2, and their acute response to 0.1 μM MTX was examined. Although cell populations from the 10 cells/cm^2 reseeding protocol behaved similar to parental HT-29 cells and underwent massive cell death, populations from the 2×10^3 cells/cm^2 reseeding protocol escaped cell death and proliferated despite exposure to the drug, though at a slightly lower rate than untreated controls (Fig. 3*e*). This experiment demonstrates that prolonged treatment of proliferating cells is *per se* insufficient to allow the emergence of acquired resistance. Instead, high cell renewal under the continuous selective pressure imposed by MTX is required.

Drug-enhanced chromosome instability contributes to the selection of resistant genetic variants

HT-29 cells are defective in the nucleotide starvation G1/S checkpoint, probably due to their *Tp53* mutant genotype (Ref. 12; data not shown). Therefore, sensitized HT-29 cells progress through the S phase in the presence of MTX, though with a marked delay (data not shown). We asked whether cell cycle events, such as centrosome duplication, would be altered during treatment. As a measure of centrosome kinetics during the cell cycle, we measured the number of mitotic spindles. HT-29 cultures growing for 2 days below the critical cell density in the presence of 0.1 μM displayed 14.0% of multipolar mitosis, similar to that found in nontreated cultures (7.2%). By contrast, treatment with conditioned medium from high-density cultures (i.e., depleted in nucleosides) and MTX dramatically increased the frequency of multipolar spindles to 79.5% (Figs. 4*a* and 4*b*). This result indicates that MTX-induced abnormal cell cycle transit through the S phase provokes centrosome reduplication with the consequent formation of abnormal spindle structures.

Moreover, MTX is known to exert clastogenic effects involving double-strand DNA breaks.[17] Accordingly, treatment of HT-29 cell cultures synchronized in the S phase led to aberrant chromosome arm fusion detected as the cells entered mitosis (Fig. 4*c*). These aberrant mitotic figures are indicative of abnormal homologous recombination between sister chromatids or nonhomologous end joining as erroneous repair of double-strand DNA breaks.

Both types of alterations, centrosome reduplication and abnormal recombination, are known mechanisms of chromosomal instability in cancer cells. Thus, we hypothesized that MTX treatment might facilitate genomic rearrangements and analyzed the karyotypes of clones shown in Figure 2. All permanently resistant clones displayed an increased number of chromosome 5q arm, either due to an increased chromosome 5 modal number or due to acquisition of a 5q isochromosome absent from the parental population (Figs. 5*a* and 5*b*; Ref. 18). Similarly, all resistant populations obtained in the experiments shown in Figure 3*d* contained an isochromosome 5q, whereas nonresistant populations displayed a chromosome pattern similar to the parental HT-29 population (Fig. 5*c*). No other apparent karyotypic abnormalities or major changes in ploidy were observed in the permanently resistant cell clones/populations. Therefore, MTX-induced increased genomic instability correlates with acquisition of resistance and involves, at least in part, 5q chromosome arm gain.

Since chromosome 5q contains the *DHFR* gene, which is often amplified in MTX-resistant tumor cells, we analyzed the *DHFR* gene dosage in the resistant clones by fluorescence *in situ* hybridization (FISH), using a genomic DNA probe containing the *DHFR* gene. The results showed no major amplification of the *DHFR* gene in any of the isolated cell populations displayed in Figure 2*a*; instead, the modal number of *DHFR* copies correlated with the number of 5q chromosome arms in each cell. The modal numbers of *DHFR* copies were as follows: nonresistant control clones 2 ($n = 3$) and 7 ($n = 4$); resistant clones 10 ($n = 7$), 11 ($n = 5$), 12 ($n = 5$), clone 13 ($n = 5$), 14 ($n = 5$) and 15 ($n = 5$). A metaphase from clone 12 is shown in Figure 5*d* to illustrate the results. Therefore, 5q chromosome gain involves genetic alterations other than *DHFR* amplification. Moreover, we analyzed the expression of DHFR protein by western blot to assess whether the slightly increased *DHFR* gene copy number resulted in increased protein levels that would explain resistance to MTX. As shown in Figure 5*e*, resistant clones displayed variable levels of DHFR protein compared with HT-29 cells, some of them having increased DHFR expression (clones 10, 14, and 15) and others diminished expression (clones 11 and 13). We thus concluded that although DHFR overexpression might be responsible for the resistant phenotype of some clones, it can not explain the resistance of other clones, which possibly acquired alterations in genes contained in chromosome 5q other than *DHFR*.

Discussion

In vitro models of drug resistance are useful to assess the mechanisms of tumor cell escape from treatment, since they allow to dissect processes of adaptation, mutation and selection under conditions of selective pressure. We have used one of such models, the HT-29 cell line and the resistance to MTX,[14] to show that the phenomenon of drug resistance acquisition is not simply the selection of resistant cells within an heterogeneous tumor cell population, but involves a complex and dynamic process where tumor genetic variants with increasing survival capacity gradually

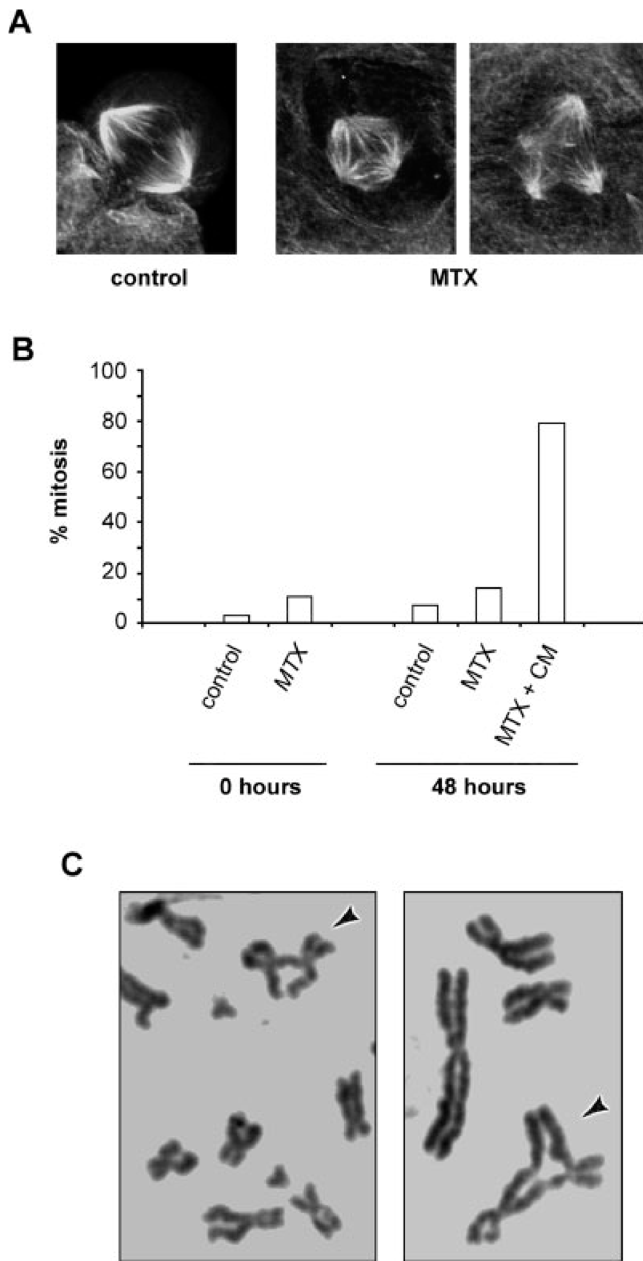

FIGURE 4 – Dynamic selection during MTX treatment involves increased genetic instability by centrosome reduplication and aberrant chromosome recombination. (*a*) Nontreated (control) and MTX-treated (MTX) HT-29 cultures grown on coverslips were processed for immunofluorescence with antibodies against α-tubulin to reveal mitotic spindles. An elevated fraction of multipolar mitosis is observed in MTX-treated cells, reflecting centrosome amplification (by the effect of MTX treatment). (*b*) Centrosome amplification was estimated from the fraction of multipolar spindles by inmunofluorescence as in (*a*); cell colonies grown at very low culture density (10 colonies/cm^2) were treated either with 0.1 μM MTX (MTX), which is a treatment situation that allows 100% cell viability, with 0.1 μM MTX as conditioned medium from cultures seeded at 2×10^4 cells/cm^2 (MTX + CM), or left untreated (control), and harvested 48 hr later. Only the treatment condition involving MTX-induced cytotoxicity (MTX + CM), but not the cell density-dependent cell survival situation (MTX), led to centrosome amplification. (*c*) Chromosome preparations from MTX-treated HT-29 cell cultures revealed the presence of abnormal mitotic recombination consistent with sister chromatid exchanges after DNA damage (arrows).

emerge and overtake more sensitive preexisting populations to give rise to full resistance.

In vitro, HT-29 cells are sensitive to low concentrations of MTX (0.1 μM) and acquire resistance to the drug upon prolonged treatment.[14] We have shown previously that when exposed to MTX for the first time, HT-29 cells initially adapt by decreasing the cell population size to a level that preserves extracellular survival factors, particularly nucleosides.[11] By elaborating further on this model, we show here that acquired resistance to MTX originates from this adaptive response in some cell clones that become independent of extracellular nucleosides for cell survival and growth. Therefore, these results allow to define 2 separable steps in the process of resistance acquisition to MTX: (*i*) preselective cell survival by means of low cell density adaptation to treatment[11] and (*ii*) emergence of stably acquired resistance.

By characterizing the kinetics of cell survival, cell growth and cell death during resistance acquisition, we describe a process designated "dynamic selection" through which permanent resistance to an antimetabolite emerges from a drug-sensitive tumor cell population. Dynamic selection takes place in a multistep process, revealing survival to MTX, and different degrees of growth capacity in the presence of MTX and survival to the combined MTX + DP treatment as hallmarks to attain full resistance. This stepwise process suggests the occurrence of separable genetic or epigenetic changes that must accumulate and lead to progressively increasing resistance. We have identified one putative event involved therein, 5q chromosome gain, which allows to distinguish all cell clones with permanent growth capacity in the presence of MTX, suggesting that 5q gain occurs at the earliest stages of the dynamic selection process. Other events may include folate binding protein α overexpression[19] among other alterations so far identified as being associated with acquired resistance to MTX.[20] We propose that these events occur sequentially in single cell clones; indeed, the heterogeneous appearance of some of them during treatment suggests that clones arise, inside clones (Fig. 1*c*). Nevertheless, only the detailed analysis of molecular events responsible for the degrees of resistance will reveal the molecular chronology of the process.

Dynamic selection involves an increased capacity of the cell population under treatment to give rise to genetic heterogeneity, which—in the continuous presence of selective pressure—may favor the emergence of the resistant genetic variants. This heterogeneity is, at least in part, due to the direct effects of MTX on the cell cycle. From work performed in parallel with the present study, we have determined that HT-29 cells progress through the cell cycle despite MTX treatment, a behavior probably related to an altered G1/S checkpoint in response to nucleotide starvation because these cells are p53 defective.[17] Although DNA replication is dramatically slowed, other cell cycle events such as centrosome duplication appear to proceed uneventfully leading to centrosome reduplication and multiple spindle formation. Since centrosome reduplication is a typical feature of aneuploid cancer cells,[21] MTX may facilitate chromosome imbalances, thus leading to the increased number of 5q chromosomes in some resistant cell populations. Moreover, MTX treatment of tumor cells defective in the nucleotide starvation checkpoint is known to lead to double-strand DNA breaks by misincorporation of uridine during DNA replication.[17,22] Although many of these breaks appear to be properly repaired (unpublished results), some may be abnormally repaired by homologous recombination with sister chromatids, e.g., leading to bridge-breakage-fusion cycles and chromosome amplification,[23,24] or by nonhomologous end joining leading to chromosome translocations or deletions.[25] In this respect, we detected an isochromosome 5q present in a proportion of resistant populations. Therefore, chromosomal alterations as a consequence of MTX effects on the cell cycle may be multiple and are expected to be involved in the resistant genetic variants originated by dynamic selection.

Gains of the 5q chromosome arm are accomplished either by an increase in modal number of chromosome 5 or by the presence of

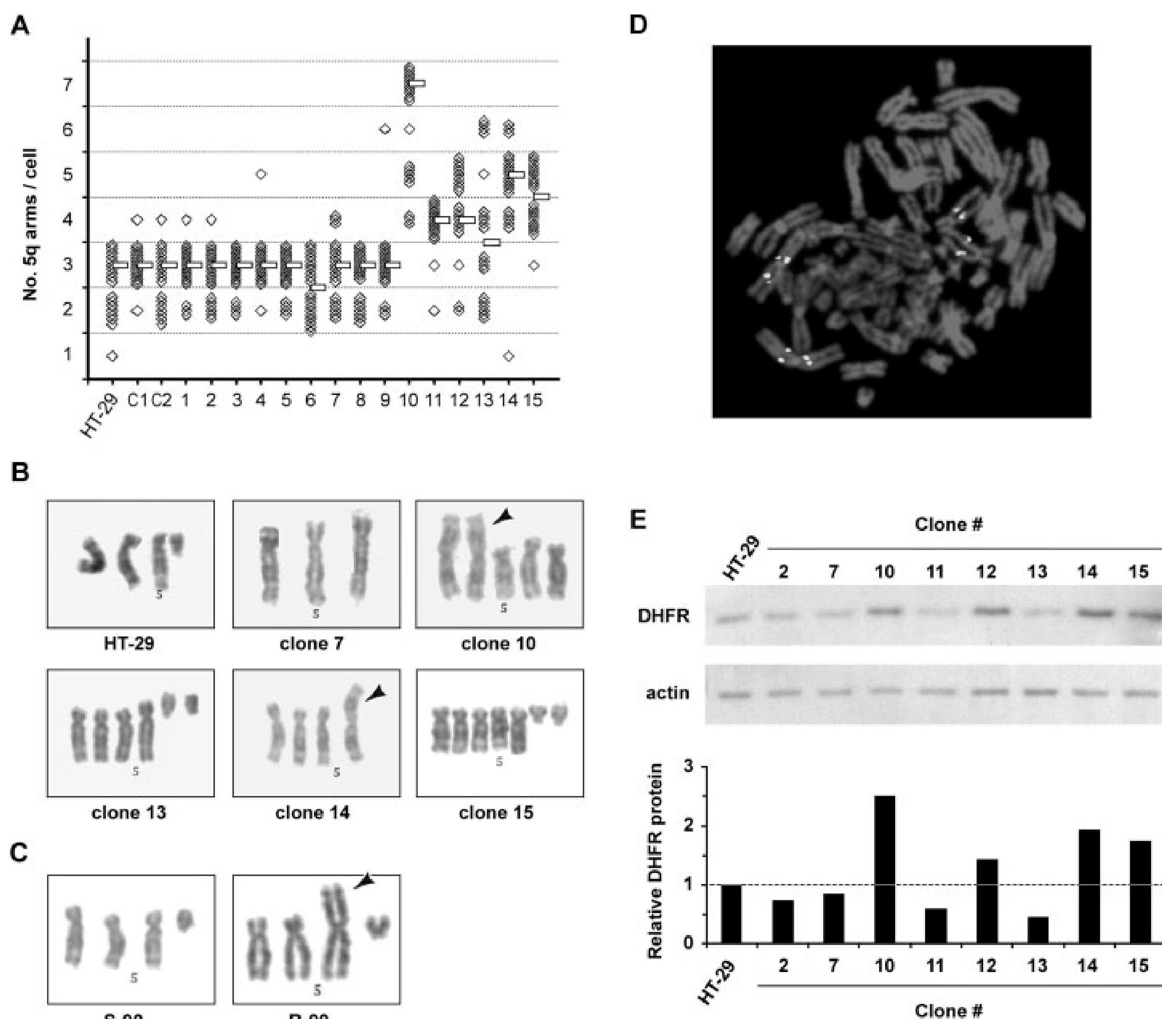

FIGURE 5 – Increased 5q chromosome dosage correlates with the acquisition of permanent resistance. (*a*) Karyotypic analysis of untreated HT-29 cells (HT-29), of 2 HT-29 cell populations isolated by clonal dilution without treatment (C1 and C2), and of the 15 cell clones described in Figure 2 (1–15). The number of 5q arms in each metaphase analyzed (white diamonds) and the 5q modal number in each cell population (white rectangles) are represented. (*b*) Photomicrographs showing representative examples of chromosome 5q arm gain in the indicated cell populations. (*c*) Photomicrographs representative from karyotypes obtained after the 9th seeding round of the experiment shown in Figure 3D, including a cell population under the 10 cells/cm^2 seeding round protocol (S-90) and a cell population from the 2×10^3 cells/cm^2 seeding round protocol (R-90). Arrows indicate the presence of an isochromosome 5q. (*d*) FISH analysis on resistant clones displaying an increased number of 5q arms revealed that there were no major changes in the *DHFR* gene, particularly ruling out *DHFR* amplification; a metaphase of clone 12 is shown illustrating *DHFR* gene signals, 4 of them on 5q isochromosomes. (*e*) Western blot analysis of DHFR protein expression in HT-29 clones (upper panel); DHFR signal was quantified with the Image Quant software (Molecular Dynamics) using α-actin for normalization, and values expressed relative to DHFR expression in the untreated HT-29 cell line (lower panel).

isochromosomes 5q. Importantly, the 5q arm contains the *DHFR* gene, which is the enzyme targeted by MTX, and *DHFR* gene amplification is a known mechanism of acquired resistance to MTX in tumor cells, including HT-29 cells treated at high doses (0.1 and 1.0 mM).[15,23] Under the conditions studied in our work, only 5q gains have been detected, possibly less than 2-fold dose increase in most cases, which might be related to the mechanism described in previous studies reporting PKCα effects on *DHFR* amplification and expression in cells resistant to 0.1 μM MTX.[26] Overt amplification of *DHFR* was ruled out by the analysis of *DHFR* copy number by FISH (Fig. 5*d*), revealing that 5q gains involved single copies of *DHFR*. Moreover, western blot analysis of DHFR expression revealed that there was no consistent trend of change in protein levels in relationship to gene copy number, consistent with previous data reporting no increases of DHFR enzyme activity in 0.1 μM MTX-resistant HT-29 cells.[15] These results suggest that other genes contained in the 5q arm might have also contributed to MTX resistance, in normal or mutated form. These issues merit further investigation.

HT-29 cells harbor the typical genetic alterations of colorectal cancer, i.e. loss-of-function of *Tp53* and *APC* tumor suppressor genes and the aneuploidy-associated chromosomal instability phenotype.[1,18] In culture, HT-29 and other colorectal cancer cells retain elevated rates of chromosomal instability, both structural and numerical, allowing clonal divergence in the absence of drug selective pressure.[27–29] However, intrinsic genetic instability is insufficient to produce variants resistant to MTX. Rather, resistance is only attained under the high cell turnover and continuous selection pressure of MTX-induced cell death (multiround treatment protocols shown in Fig. 3*d*). Whether this behavior reflects the importance of cell turnover, of MTX-induced damage, or both can not be directly inferred from our results. Interestingly, the importance of cell turnover is in agreement with the notion that selection is at least as important as mutation to drive the progres-

sion of cancer cells during carcinogenesis.[30] Further analysis of the dynamic selection model considering different types of genomic instability as well as other types of selection pressure may help to understand how these mechanisms operate during acquisition of drug resistance.

It is presently unclear whether the *in vitro* determinants that underlie these population dynamics also occur in tumors.[11] Therefore, *in vivo* experiments should be designed to address this issue. The fluctuating depletion of extracellular nucleosides during continuous MTX treatment *in vitro* imposes increased cell turnover as a driving force of dynamic selection. This is reminiscent of the changes within the tumor microenvironment undergoing tumor progression. In particular, avascular tumor micrometastasis undergoes similar balanced cell proliferation and apoptosis in response to angiogenic and antiangiogenic factors, which impose the selection pressure required for the emergence of genetic variants resistant to hypoxia.[31,32] Increased cell turnover may also occur in the presence of an inflammatory or immune response in tumors.[33] Therefore, our results provide an *in vitro* model for the study of the tumor cell population dynamics resulting from selective pressure that might be useful to uncover the complex sequence of molecular events involved in tumor progression.

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