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Inhibition of Cell Growth and Polyamine Levels in Lymphocytes Caused by the Combination of Methotrexate and Chloroquine

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SUMMARY. The objective of this study was to investigate the effects of the combination of methotrexate (MTX) and chloroquine (CQ) on cell growth and polyamine levels for lymphocyte and lung fibroblast cells. The action of CQ inhibited cell growth in the first 24 h for both cell lines. The combination of the drugs decreased polyamine levels, affecting protein synthesis and cell growth for the lymphocyte cells. These results are important for rheumatoid arthritis because the combination of the drugs acts mainly on lymphocyte cells, which are the agents responsible for joint inflammation and the erosion of cartilage.

RESUMEN. "Inhibición del Crecimiento de las Células y de los Niveles de Poliamina en Linfocitos Causada por la Combinación del Metotrexato y de la Cloroquina" . Fue realizado un estudio utilizando dos líneas celulares de linfocitos y fibroblastos pulmonares, con el objeto de investigar el efecto conjunto del metotrexate (MTX) y la cloroquina (CQ) sobre el crecimiento celular y los niveles de poliamina. Los resultados muestran que la CQ inhibió el crecimiento de ambas líneas tan sólo durante las primeras 24 h, mientras que la combinación de ambas drogas disminuyó los niveles de poliamina que afectan la síntesis proteica y el crecimiento celular, sólo en los linfocitos. Estos resultados son relevantes en la artritis reumatoidea, ya que la combinación de ambas drogas actúa principalmente sobre los linfocitos, responsables de la inflamación de la articulación y la erosión del cartílago.

INTRODUCTION

Several combinations of medicines are used in the treatment of rheumatoid arthritis (RA). In some cases the combined drugs are intended to potentiate each others' effects; in other cases they prevent fast elimination or interfere in the metabolism of one of the medicines. The most successfully used combination is methotrexate (MTX) and non-steroid anti-inflammatory drugs (NSAIDs), but a high incidence of hepatotoxicity related to the dose or prolonged use of MTX is frequently encountered [1].

Various authors have suggested that the combination of MTX and Chloroquine (CQ), in addition to being an efficient therapy, reduces MTX-related hepatotoxicity [2-4]. The mechanisms by which the combination of MTX and CQ mitigates the hepatotoxic effects of MTX are unknown.

The mechanism of MTX action has been related to the inhibition of dihydrofolate reductase (DHFR), which results in the inhibition of protein synthesis and the blockage of methionine regeneration from homocysteine, leading to a depletion of S-adenosylmethionine (SAM) and polyamines (putrescine, spermidine and spermine). A decrease in polyamine levels results in anti-proliferative and immunomodulatory effects [5].

The mechanism of CQ action, as an immune modulator, is mainly based on the functional impairment of intralysosomal, by elevating the intracellular pH [6]. Additionally, CQ inhibits histamine and putrescine catabolism by blocking histamine N-methyltransferase (HNMT) [7] and diamino oxidase (DAO) [8] activities , respectively.

HNMT is dependent on methylation donated by SAM, and an accumulation of histamine reduces hepatic alanine and aspartate transami-

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nases (ALT and AST) and protects the liver against the main liver-injury experimental models: lipopolysaccharide [9], alcohol [10] and carbon tetrachloride [11].

The inhibition of putrescine catabolism by CQ leads to an accumulation of putrescine and the relevance of this accumulation is not clear, but an increase in polyamine levels in fast-growth cells and tissues, such as cancer cells, and in the urine of rheumatoid arthritis patients has been observed [12,13].

Since MTX or CQ alone affect polyamine biosynthesis, it was investigated whether chloroquine interferes in the anti-proliferative effects and polyamine levels of cells treated with MTX. Such results would explain, at least in part, the relevance of this interaction to the therapeutic and toxic effects demonstrated in rheumatoid arthritis patients.

MATERIALS AND METHODS

Cell Lines and Cultures

The effects of MTX and CQ were tested on two different cell lines: 2B4 lymphocytes (suspension cells) and V79 lung fibroblasts (adherent cells). Murine hybrid 2B4 lymphocyte cells were cultured in RPMI 1640 medium and V79 lung fibroblast cells were cultured in DMEM medium. All mediums were supplemented with 10% FCS, 2 mM L-glutamine, and either 50 U/ml streptomycin, or 100 U/ml penicillin G or gentamicin. The cells were cultured at 37 °C in an atmosphere of 5% CO_2 for 72 h at starting concentrations of $1x10^4$ cells/ml. Cell growth was quantified by MTT assay or by dye exclusion with 0.5% Trypan Blue.

Polyamine Measurement

All cell suspensions ($6x10^5$ cells/ml) were harvested and extracted with 0.3 ml of 5% trichloroacetic acid. A supernatant was then obtained by centrifugation and used in the polyamine assays. The polyamines (putrescine, spermidine and spermine) were measured by High Performance Liquid Chromatography (HPLC).

Statistical Analysis

The cell culture data was submitted to an ANOVA one-way test.

RESULTS

The doses of MTX alone required to inhibit cell growth for 72 h (70-90% inhibition compared to non-treated cells) were much smaller for 2B4 cells (8 nM) compared to adherent V79 cells (150 nM). Cell division time (every 6 h) stopped completely after 48-72 h of treatment with MTX (Fig. 1A). This data shows that the inhibitory effects of MTX can be observed only after 24 h, and that the effect on the different lineages of cells is related to the doses used.

The CQ doses required to inhibit 2B4 and V79 cells during incubation for 72 h were 3 µM and 40 µM, respectively. At these doses, CQ delayed the growth rate of 2B4 cells from 6 to 9 h, and completely stopped the growth of V79 cells, for up to 24 h. After this time, the growth rate of both cell lines followed similar patterns to those of the non-treated cells when they were subsequently incubated for 48 and 72 h (Fig. 1B). Increases in CQ doses intended to prolong growth inhibition induced death for all cells (data not shown).

The combination of MTX and CQ delayed the growth rate from 6 to 12 h for 2B4 cells for up to 48h, and completely stopped growth from 48 to 72 h. For V79 cells, the growth rate was

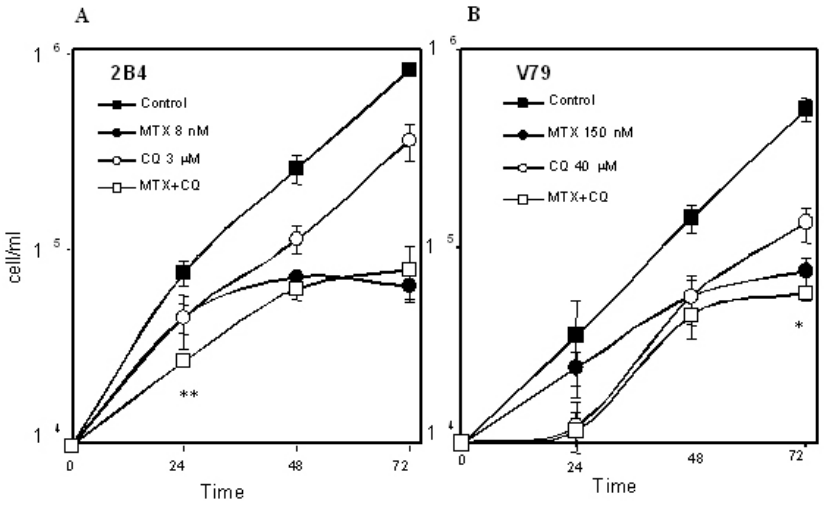

Figure 1. Inhibition of cell growth by treatment with MTX, CQ and MXT plus CQ. The 2B4 (**A**) and V79 (**B**) cells were cultured with methotrexate (MTX) or Chloroquine (CQ) alone or MTX plus CQ for 72h. The concentrations used were: 8nM MTX and 3 µM CQ for 2B4 cells and 150 nM MTX and 40 µM CQ for V79 cells. Each point represents the mean ± standard deviation of triplicate determinations of cell number. * Significant difference ($p<0.05$) from the control group. ** Significant difference ($p<0.05$) from the control and CQ groups.

	Polyamines (nmol / mg Protein)		
	Putrescine	**Spermidine**	**Spermine**
2B4 cells			
Control	2.76 ± 0.25	26.07 ± 0.36	10.35 ± 0.51
MTX 8 nM	3.22 ± 0.24	18.58 ± 1.98*	9.41 ± 1.53
CQ 3 µM	4.62 ± 0.34	23.56 ± 0.84	8.97 ± 0.39
MTX + CQ	1.68 ± 0.07*†	15.59 ± 0.65*	10.88 ± 0.90
V79 cells			
Control	1.99 ± 0.22	24.70 ± 3.87	7.91 ± 1.01
MTX 150 nM	2.53 ± 0.85	22.50 ± 0.56	9.20 ± 2.03
CQ 40 µM	2.89 ± 0.27	25.90 ± 0.43	8.55 ± 1.45
MTX + CQ	3.01 ± 0.78	21.20 ± 2.92	8.98 ± 1.23

Table 1. Polyamine content in cells treated with methotrexate, chloroquine, and methotrexate plus chloroquine. Cells were cultured with Methotrexate (MTX), Chloroquine (CQ) or MTX + CQ for 72 h, and polyamine contents were determined by High Performance Liquid Chromatography (HPLC). Values are the means ± Standard Deviations of triplicate determinations. * Significant difference ($p<0.05$) from the control group. † Significant difference ($p<0.05$) from the MTX + CQ group.

no different to that of CQ alone up to 48 h, but stopped completely from 48 to 72 h, showing the same behaviour as observed for 2B4 cells. All cells died after subsequent incubation for 96 or 120 h, even when they were transferred to a new medium without the drugs.

The polyamine content was not affected in V79 cells when treated with MTX, CQ or the combination of the drugs. Conversely, a significant reduction in putrescine and spermidine levels was observed in the 2B4 cells treated with MTX or MTX plus CQ compared to the control group. The decrease in the putrescine levels of the cells treated with the combination of MTX and CQ was more pronounced than that observed for MTX alone (Table 1).

DISCUSSION

The results show that CQ has a fast inhibitory onset against cell growth and does not affect the inhibition caused by MTX during the remaining incubation time. The antiproliferative effect of CQ seems to be caused by intralysosomal alcalinization, as it was effective only in the first 24 h for both cells lines studied. This effect suggests that the cells have a special pump to eliminate the CQ and restore intracellular pH, since after 48h of incubation, cell growth was restored to the same rate as that of the non-treated cells. This pumping out of CQ may occur through P-glycoprotein and MRP membrane transporters [15,16].

From the therapeutic point of view, this result is of interest for the RA therapeutic schedule and explains, in part, the daily requirement of CQ and the effectiveness of the combination of MTX and CQ, as the cell growth inhibition action of CQ starts immediately and is then followed by the delayed action of MTX.

Even though the majority of the CQ is pumped out of the cells within 24 h, it could be seen that the remainder still maintains its effects for up to 72 h in 2B4 cells, because an additive effect with MTX was observed in the decreasing of polyamine levels.

2B4 cells are suspension cells and their uptake of MTX is faster and higher than in adherent cells, so this rate of uptake maybe relevant to affect SAM and polyamine inhibition, as can be seen in the results reported by Kimura *et al.* [17], where the growth of adherent cells were inhibited but SAM and polyamine levels were not altered.

According to the results of the present study, the combination of a low dose of MTX and CQ affects the suspension cells first and strongly, and the combination is therefore important for the inhibition of lymphocyte proliferation via polyamines methylation pathways.

The lack of effect of this combination on adherent cells suggests that growth inhibition in these cells is due to purine and pyrimidine biosynthesis blockage, and that the S-adenosylmethionine or polyamines methylation pathways are not involved.

From these results, it can be concluded that the combination of MTX and CQ is effective for the control of lymphocyte proliferation and is a suitable therapeutic prescription for RA.

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