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European Journal of Pharmacology 316 (1996) 349–357

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Eicosanoid inhibitors enhance synergistically the effect of transforming growth factor β1 on CCL 64 cell proliferation

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Received 17 April 1996; revised 10 July 1996; accepted 23 August 1996

Abstract

The interactions between drugs suppressing the production of arachidonic acid metabolites-eicosanoids and transforming growth factor β1 (TGF-β1) were investigated using CCL64 cells. These experiments, designed as complete factorial combination of treatments, demonstrated that both esculetin and eicosatetraynoic acid significantly potentiated the inhibitory effect of TGF-β1 on [^{3}H]thymidine incorporation. The expression of overadditive effects depended both on the type and concentration of combined factors. These results corresponded with cell cycle analysis data (increased cell number in G_1 and decreased cell number in S and G_2/M phases) and with the results monitoring cell number following treatment with eicosatetraynoic acid, esculetin, 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid (MK-886) and indomethacin. Summarizing, the degree of significance of combined effects supports the hypothesis of synergistic potentiation of TGF-β1 effects caused by eicosanoid inhibitors. The results indicate that either the lack of some eicosanoids or a certain type of misbalance in the metabolism of arachidonic acid leading to its abundance might modulate TGF-β1 effects on the cell cycle and proliferation in CCL64 cells.

Keywords: Arachidonic acid; TGF-β1 (transforming growth factor β1); Epithelial cells; Cell cycle; Cell proliferation

1. Introduction

Transforming growth factor β (TGF-β) represents a family of growth factors that appear to be intimately involved in the regulation of many physiological and pathological processes such as cell proliferation and differentiation (Roberts and Sporn, 1988; Barnard et al., 1990), embryogenesis (Roberts et al., 1990), immune response (Ahuja et al., 1993), apoptosis (Teatle et al., 1993) and carcinogenesis (Roberts et al., 1988). TGF-β1 is the prototype of this family. While TGF-β1 has a stimulatory influence upon many cells of mesenchymal origin, for many other cell types, especially epithelial-like-cells, TGF-β1 is an inhibitor of cell cycling. Although TGF-β1 is one of the most intensively studied growth regulators, the precise mechanism(s) by which it mediates the cell cycle arrest is not completely understood (Johnson, 1994). The mechanism of activation of transmembrane serin/threonine kinases called TGF-β1 receptors I and II has been relatively well documented (Lin et al., 1992; Attisano et al., 1994). Some effects of TGF-β1 might be due to differences in a particular ability of cells to activate the latent form of TGF-β1 which is secreted, or due to differences in postreceptor mechanisms (Roberts and Sporn, 1988; Liu and Oliff, 1991; Yan et al., 1994). Recent evidence indicates that inhibition of G_1/S transition induced by TGF-β1 is the result of TGF-β1 ability to regulate expression and/or activity of cyclin dependent kinases, cyclins and cyclin dependent kinase inhibitors (Geng and Weinberg, 1993; Attisano et al., 1994; Ko et al., 1995).

Recently, an increased interest has appeared in the study of the role of membrane polyunsaturated fatty acid, arachidonic acid (Naor, 1991; Sumida et al., 1993), and its metabolites, eicosanoids, especially products of lipoxygenases, in the mechanisms of cytokine signal transduction pathways (Peppelenbosch et al., 1992; Masongarcia et al., 1992; Bandyopadhyay et al., 1993; Miller et al., 1993). Using various lipoxygenase inhibitors, it has been shown by us and others, that an intact lipoxygenase pathway is necessary for normal mouse and human myelopoiesis both in vitro (Miller et al., 1986; Kozubík et al., 1994) and in

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PII S0014-2999(96)00691-7

vivo (Kozubík et al., 1993; Kozubík et al., 1994), as well as for the proliferation of human leukemic cell lines (Snyder et al., 1989). These results indicate that endogenous non-cyclooxygenase metabolites of arachidonic acid could act as intracellular mediators of mitogenic signals.

Therefore, it is surprising, that there is very little information about the relationships of arachidonic acid metabolites and TGF-β1 signalling. It may be hypothesized that arachidonic acid themselves or some of their products, eicosanoids, may be responsible for mediating of growth inhibition induced by TGF-β1. The aim of experiments reported here was to investigate the ability of inhibitors of arachidonic acid metabolism, i.e., 6,7-dihydroxycoumarin (esculetin), 5,8,11,14-eicosatetraynoic acid, 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid (MK-886) and indomethacin to modify the antiproliferative effect of TGF-β1. We tested the effects of these inhibitors and their combinations with TGF-β1 on [^{3}H]-thymidine incorporation, cell cycle and growth of the CCL64 mink lung epithelial cell line. CCL64 cells are extremely sensitive to TGF-β1 and are currently used for TGF-β1 bioassays (Garrigue-Antar et al., 1995) and for studies of TGF-β1 signal transduction (Mazars et al., 1995). Our results indicate that the inhibitors of arachidonic acid metabolism significantly strengthened the inhibitory effects of TGF-β1 on the cell cycle and proliferation of CCL64 cells.

2. Material and methods

2.1. Cell line

CCL64 mink lung epithelial cells were maintained in Dullbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum and gentamycin (50 μg/ml) at 37°C in humidified incubator with 5% CO_2.

2.2. Drugs

Esculetin (Sigma, St. Louis, MO, USA), an inhibitor of 5-lipoxygenase (IC_{50} = 4 μM) and 12-lipoxygenase (IC_{50} = 2.5 μM), does not inhibit cyclooxygenase (Neichi et al., 1983); 5,8,11,14-eicosatetraynoic acid (Sigma), a competitive analogue of arachidonic acid with pleiotropic effects (Tobias and Hamilton, 1979), inhibits cyclooxygenase (IC_{50} = 8 μM), 5-, 12-, 15-lipoxygenases (IC_{50} 10; 0.3; and 0.2 μM, respectively), phospholipase A2 and cytochrome P450; indomethacin, an inhibitor of cyclooxygenase (IC_{50} = 0.1 μM) (for detailed description of these agents and their actions see also BIOMOL Research Laboratories, Catalogue 1992); MK-886 (a gift from Merck, Canada), a specific inhibitor of 5-lipoxygenase activating protein prevents leukotriene formation in vivo and in intact cells in vitro (Rouzer et al., 1990). The drugs were diluted in absolute ethanol and added to the cultivation medium. The final concentration of ethanol did not exceed 0.1%, the concentration which did not influence any of the parameters tested. TGF-β1 (Sigma) was prepared as a stock solution (1 μg/ml) in HCl-bovine serum albumin and diluted in the cultivation medium.

2.3. Detection of the cell cycle and cell proliferation

10^5 cells/ml were seeded in 24 well plates in DMEM with 5% fetal calf serum and incubated for 8 h. Subsequently, the medium was replaced by DMEM with 1% fetal calf serum and incubated for a further 20 h. Appropriate drugs were then added and cells were cultivated for further 24 h (for determination of cell cycle parameters and incorporation of [^{3}H]thymidine) or 48 h (for determination of cell number). After this time the cells were trypsinized, collected and evaluated as described in following sections.

2.3.1. [^{3}H]Thymidine incorporation

[^{3}H]Thymidine (3.7 kBq/ml) was added for last 2 h of cell cultivation and its incorporation into DNA-synthesizing cells was measured by liquid scintillation counting. The experiments were independently repeated at least 5 times in 6 parallels.

2.3.2. Flow cytometry

Collected cells were cooled in ice, washed with cold phosphate buffered saline and stained with propidium iodide in Vindelov's solution (Vindelov, 1977) for 30 min in 37°C. Fluorescence (DNA content) was measured with Coulter EPICS XL apparatus (using argon ion laser at 488 nm for excitation). $(1-2) \times 10^4$ cells were analyzed for each sample and percentage of cells in each phase of the cell cycle was determined using standard software. Three independent experiments were performed.

2.3.3. Cell number determination

Cell number was counted using Coulter Counter (model ZM) after 48 h of cultivation. Two independent experiments in three parallels were performed.

2.3.4. Cell viability

Cell viability was determined microscopically using trypan blue (0.4%) exclusion assay. The number of viable (unstained) cells was expressed as percentage of total number (200) of counted cells.

2.4. Experimental design and data analysis

The statistical analyses were performed using SPSS/PC + Version 4.0 program (SPSS/PC + Version 4.0 Base Manual for IBM). All data represent the mean values and standard errors of mean (S.E.M.) of normal or binomial distributions depending on type of analysis.

2.4.1. [^{3}H]Thymidine incorporation

The experiments were designed as complete factorial combination of treatments. Experiment I tested the combinations of TGF-β1 (0.125–0.5 pM) with esculetin (5–20 μM) and experiment II realized the combinations of TGF-β1 (0.125–0.5 pM) with eicosatetraynoic acid (1.25–10 μM). The results of both experiments were analyzed using standard two-way analysis of variance (ANOVA; fixed model) after preliminary tests of normality and homogeneity of variance. The Newman–Keuls multiple-range test was used for the comparison of differences between the means of experimental variants taking $P = 0.05$ as the limit of probability (Zar, 1974). The ANOVA models were used to assess the significance of fixed effects of single factors as well as their interaction. The null hypothesis for an interaction between two combined factors was that the response of [^{3}H]thymidine incorporation did not differ among specific levels of one factor depending upon the particular level of the second factor. When the ANOVA showed a significant interaction between the factors, the general approach in investigating the regression model from two-way experimental data was applied (Anderson and McLean, 1974).

The [^{3}H]thymidine incorporation data was used to construct third order polynomial models involving both single effects of TGF-β1, esculetin and eicosatetraynoic acid as independent variables and also their interactive terms. The quantitative contribution of interactive and additive regression components to the total inhibition effect was assessed separately for each combination of factors using the constructed regression models. Response surfaces were modelled by fitting untransformed data to polynomials. The evaluation of regression models was based on the correlation between observed and predicted [^{3}H]thymidine incorporation levels, the lack-of-fit statistic (F-test), and the significance of estimated parameters.

All the results were presented as a percentage of [^{3}H]thymidine incorporation level in control samples.

2.4.2. Cell cycle analysis

Relative proportions of cell numbers in discrete phases of cell cycle (flow cytometric analysis) were evaluated using binomial parameter p and the effects of experimental treatments were compared on the basis of two sample binomial test. Replicated flow cytometric experiments were combined under assumption of homogeneity of binomial distributions (Zar, 1974). For statistical evaluation of the data obtained the number of cells in G_1 phase and number of cells in S and G_2/M phases (proliferating part of population) were used.

2.4.3. Cell number determination

Counts of cells as affected by experimental treatments were compared using non-parametric Mann–Whitney test due to non-homogeneity of variance and departures from normality (Zar, 1974).

3. Results

We have carried out our studies in the medium containing 1% serum, because cellular sensitivity to TGF-β1 increases about five times as compared with 5% serum (Garrigue-Antar et al., 1995; our own observation). This serum concentration did not affect proliferation of control CCL64 cells during the time period tested. Under these conditions the viability of cells treated with selected drugs and their combinations did not decrease below 90% in all types of our experiments.

The effects of single agents (TGF-β1, esculetin, and eicosatetraynoic acid) on [^{3}H]thymidine incorporation into CCL64 cells are presented in Fig. 1. All the agents used decreased [^{3}H]thymidine incorporation in a dose-dependent manner. The dose–response curve of TGF-β1 appears to be steeper in comparison with eicosatetraynoic acid and esculetin, considering the range of experimental concentrations.

Based on these data, a wide range of concentrations of inhibitors of arachidonic acid metabolism decreasing the cell response in the range of 10 to 50% (esculetin) and of 30% to 70% (eicosatetraynoic acid), approximately, were combined with relatively less efficient concentrations of TGF-β1: 0.125 pM, threshold dose; 0.25 pM, 5% of inhibition; 0.5 pM, 20% of inhibition of [^{3}H]thymidine incorporation. Mean values illustrating the effects of some of these combinations and their standard deviations are shown in Fig. 2 and Fig. 3. The results demonstrated that both esculetin and eicosatetraynoic acid strengthened the inhibitory effect of TGF-β1 on [^{3}H]thymidine incorporation. For a precise statistical evaluation of both experiments, stepwise multiple regression and regression on all possible subsets of independent parameters identified equa-

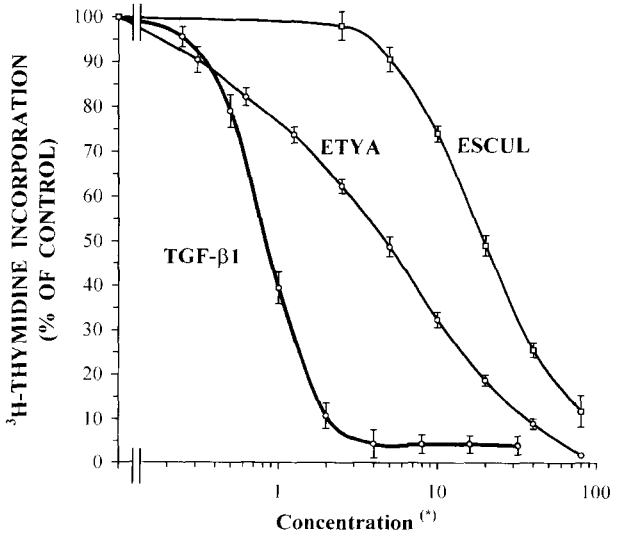

Fig. 1. The effects of TGF-β1, esculetin (ESCUL) and eicosatetraynoic acid (ETYA) on [^{3}H]thymidine incorporation into CCL64 cells. (*) Concentration of factor in pM (TGF-β1) or in μM (ETYA, ESCUL).

Table 1
Parameter estimates of regression models describing [^{3}H]thymidine incorporation (% of control), based on the variables TGF-β1, esculetin (ESCUL) and eicosatetraynoic acid (ETYA)

Model I: TGF-β1 and ESCUL [a]						Model II: TGF-β1 and ETYA [b]					
Variable	Parameter estimate [c]	Standard error	Significance level	Multiple correlation [d]	LOF [e]	Variable	Parameter estimate [c]	Standard error	Significance level	Multiple correlation [d]	LOF [e]
Intercept	100.00	1.13	0.000			Intercept	100.00	1.13	0.000		
TGF-β1	−3.61	1.32	0.002			TGF-β1	−3.61	1.09	0.002		
TGF-$\beta1^2$	−81.83	4.97	0.000			TGF-$\beta1^2$	−81.83	4.54	0.000		
ESCUL	−2.72	0.12	0.000	0.931	0.114	ETYA	−26.07	3.98	0.000	0.939	0.159
$ESCUL^2$	0.012	0.001	0.009			$ETYA^2$	4.31	0.51	0.000		
(TGF-β1)·(ESCUL)	−19.21	3.56	0.001			$ETYA^3$	−0.24	0.02	0.003		
(TGF-β1)·($ESCUL^2$)	2.04	0.57	0.002			(TGF-β1)·(ETYA)	−17.09	3.62	0.000		
(TGF-β1)·($ESCUL^3$)	−0.057	0.019	0.005			(TGF-$\beta1^2$)·(ETYA)	25.05	5.42	0.003		

[a] $\hat{Y} = a_0 + a_1(\text{TGF-}\beta1) + a_2(\text{TGF-}\beta1^2) + a_3(\text{ESCUL}) + a_4(\text{ESCUL}^2) + a_5(\text{TGF-}\beta1)(\text{ESCUL}) + a_6(\text{TGF-}\beta1)(\text{ESCUL}^2) + a_7(\text{TGF-}\beta1)(\text{ESCUL}^3)$.
[b] $\hat{Y} = a_0 + a_1(\text{TGF-}\beta1) + a_2(\text{TGF-}\beta1^2) + a_3(\text{ETYA}) + a_4(\text{ETYA}^2) + a_5(\text{ETYA}^3) + a_6(\text{TGF-}\beta1)(\text{ETYA}) + a_7(\text{TGF-}\beta1^2)(\text{ETYA})$.
[c] Estimates are scaled to express $\hat{Y}$ as percent of control.
[d] Simple correlation between observed (Y) and predicted ($\hat{Y}$) values.
[e] Significance probability for lack-of-fit F statistic (significance level).

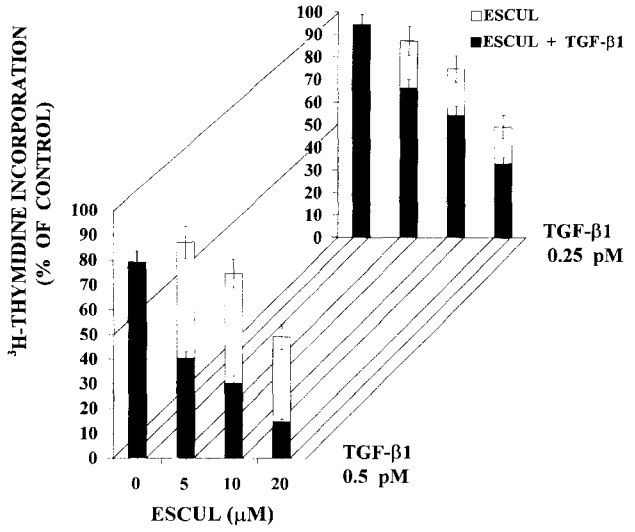

Fig. 2. The effect of selected combinations of TGF-β1 and esculetin (ESCUL) on [^{3}H]thymidine incorporation into CCL64 cells (Exp. I).

tions including the best variables for the cubic polynomial model of the data. The variables included in the models are specified in Table 1. Both the combination of TGF-β1 with esculetin (Exp. I) and TGF-β1 with eicosatetraynoic acid (Exp. II) caused highly significant ($P = 0.001$) over-additive inhibition of [^{3}H]thymidine incorporation. These interactive effects led to the incorporation of interactive components in the regression models (Table 1; model I: TGF-β1 × esculetin, TGF-β1 × esculetin^2, TGF-β1 × esculetin^3; model II: TGF-β1 × eicosatetraynoic acid, TGF-β1^2 × eicosatetraynoic acid). Regression models including more variables than those given in Table 1 resulted in only a slightly improved fit to the data. The F-tests indicated a non-significant lack-of-fit between the polyno-

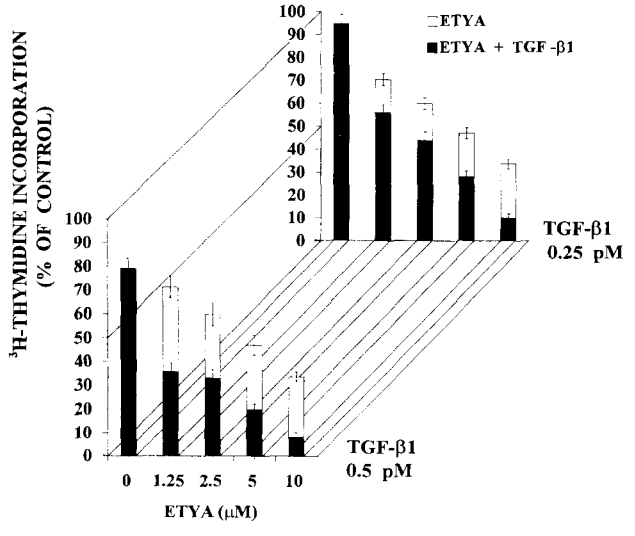

Fig. 3. The effect of selected combinations of TGF-β1 and eicosatetraynoic acid (ETYA) on [^{3}H]thymidine incorporation into CCL64 cells (Exp. II).

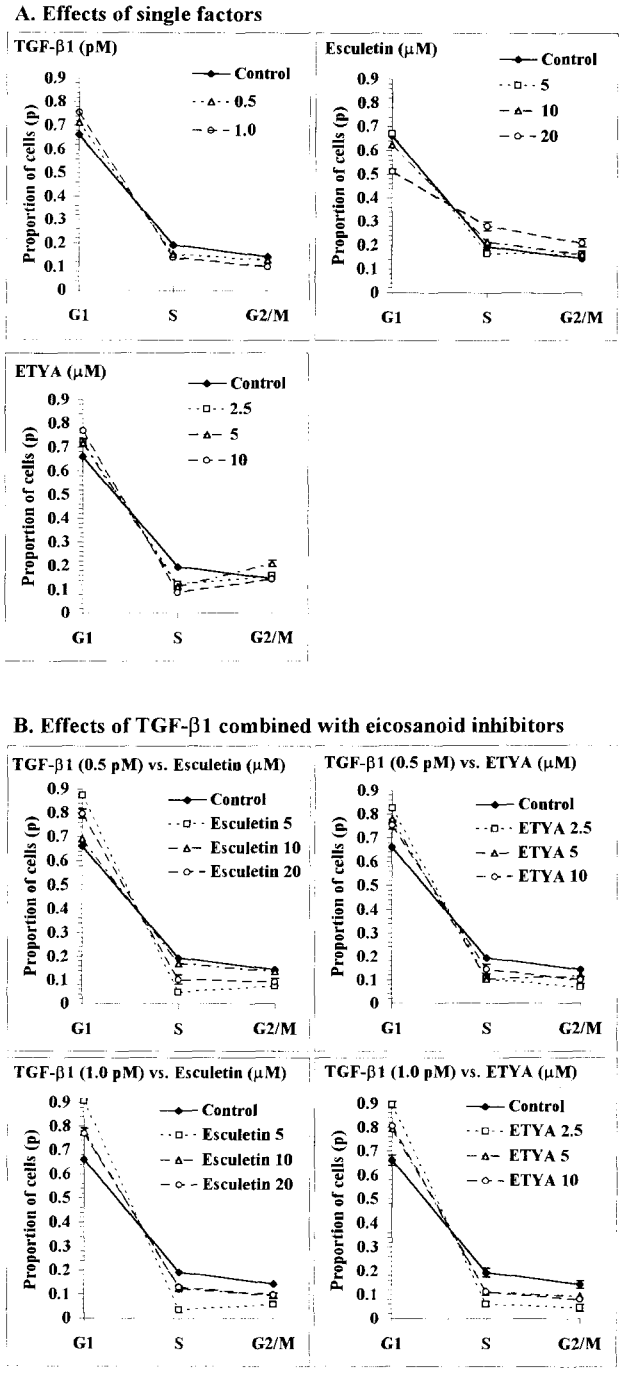

Fig. 4. The distribution of cells in discrete phases of the cell cycle upon the treatment with (A) TGF-β1, esculetin, and eicosatetraynoic acid (ETYA), and (B) their combinations. Results are weighed averages of three independent experiments (weighed with respect to the cell number involved in flow cytometric analysis; $(1-2)\times 10^4$ cells per sample were measured). Due to space limitation, standard errors of binomial parameter (p) are shown in selected points. The least significant difference for comparison of treatments was evaluated in the range 0.041–0.056 (binomial tests; significance level: $P = 0.01$). Proportion of cells in discrete phases of the cell cycle and their changes are visualized using the lines connecting the discrete data points.

mial models and the data for both experiments, which is a further evidence of the reliability of the models.

On the basis of model evaluation of experimental data (Table 1) we can conclude that the effects of non-interactive components increased with an increasing dose of esculetin within all doses of TGF-β1. The increasing contribution of interactive components was related to the increasing concentrations of TGF-β1 for all concentrations of esculetin tested. Based on the range of tested concentrations, it appeared that the quantitative portion of the interactive effect in the total inhibition effect decreased with an increased dose of esculetin. However, there was no significant difference in the potentiation of TGF-β1 by esculetin in the middle range of concentrations of esculetin.

Similarly, the model analysis of data (Table 1) showed that the effects of non-interactive components increased with an increasing dose of eicosatetraynoic acid within all doses of TGF-β1. The contribution of model interactive components was directly proportional to the dose of eicosatetraynoic acid within all concentrations of TGF-β1 tested. The quantitative portion of the interactive effect in the total inhibition effect increased directly with the increased dose of eicosatetraynoic acid within a single dose of TGF-β1. An overadditive inhibition effect of TGF-β1 in combinations with high doses of both esculetin and eicosatetraynoic acid was less quantitatively apparent, mainly due to the relatively high total inhibition of cell response caused by higher doses of eicosatetraynoic acid or esculetin alone (more than 60% of inhibition).

The effects of TGF-β1, esculetin, eicosatetraynoic acid and their combinations on the cell cycle parameters were analyzed in other independent experiments as described in more detail in the legend to Fig. 4. In comparison with control (nontreated) cells, both TGF-β1 at concentrations of 0.5 and 1 pM and eicosatetraynoic acid at concentrations 2.5, 5 and 10 μM significantly lowered the amount of cells in S and G_2/M phases (i.e., the proliferating part of cell population) and enhanced the amount of cells in G_1 phase of the cell cycle if used as single agents. On the other hand, esculetin in concentrations of 5 and 10 μM influenced the cell cycle parameters only slightly. A concentration of esculetin of 20 μM increased significantly the number of cells in S and G_2/M phases and decreased their amount in G_1 phase of the cell cycle (Fig. 4A). In the combined treatment of cells with TGF-β1 and eicosanoid inhibitors a significant decrease of cell number in S and G_2/M phases and an increase of cell number in G_1 phase of the cell cycle was observed (compared with the effects of single agents). The highest effects were achieved after combined treatment with 0.5 or 1 pM TGF-β1 and 5 μM esculetin or 2.5 μM eicosatetraynoic acid. There were approximately 85–90% of cells in G_1, 3–9% in S and 4–10% in G_2/M phases (Fig. 4B). The results achieved with concentrations of TGF-β1 lower than 0.5 pM combined with both eicosanoid inhibitors were of lower statistical value (data not shown).

Moreover, the effects of 48 h treatment of CCL64 cells with single drugs and their combinations on the cell num-

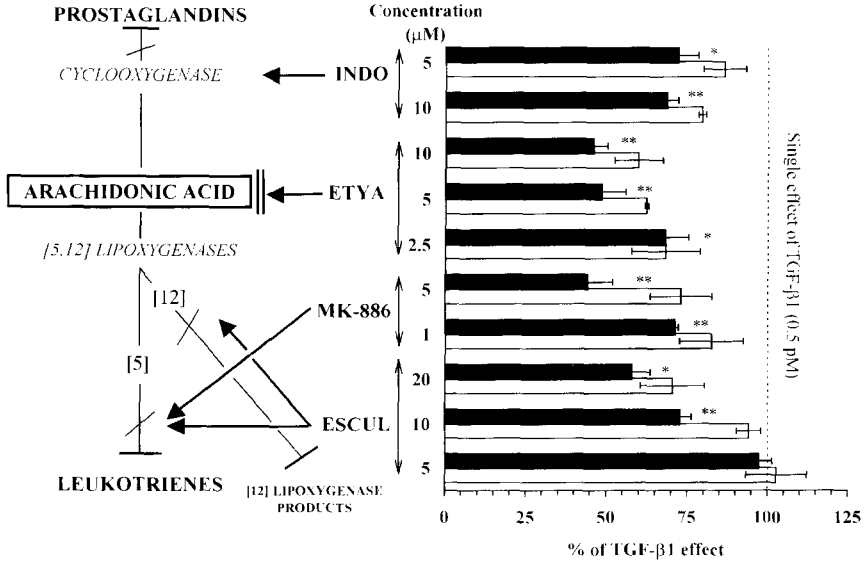

Fig. 5. Number of cells after 48 h treatment with indomethacin (INDO), eicosatetraynoic acid (ETYA), MK-886 and esculetin (ESCUL) applied as single factors (white columns) or in combination with TGF-β1 (black columns). All values are expressed as % of TGF-β1 (0.5 pM) effect. (*) Significantly lower number of cells were detected in a combination of factors comparing with single effect of TGF-β1 (Mann–Whitney test: $P < 0.05$). (**) Significantly lower number of cells were detected in a combination of factors comparing with single effect of both TGF-β1 and the other factor involved in the mixture (Mann–Whitney test: $P < 0.05$). The expected inhibiting effects on the pathways of arachidonic acid metabolism are expressed on the left side of the picture.

ber were evaluated in other independent experiments. After this time TGF-β1 (0.25–4 pM), acting as a single agent, decreased the number of cells up to 60% of control depending on the dose (significantly from 1 pM — 75% of the control).

In this experimental arrangement the doses which affected the cell number only marginally, i.e., 0.25 pM (90% of control value) and 0.5 pM (86% of control value) TGF-β1, were chosen for the combined treatment. Fig. 5 shows cell number after 48 h of treatment with a broader spectrum of inhibitors of arachidonic acid metabolism (indomethacin, eicosatetraynoic acid, MK-886 and esculetin) and their combinations with 0.5 pM TGF-β1. With the aim to better describe the effects of mixtures the results were expressed as a percentage of effects of TGF-β1 alone. It was shown that all the inhibitors if acting as single factors (except 5 μM esculetin) were able to decrease the number of cells in comparison with the effects of TGF-β1 alone (= 100%) and that these effects were significantly (and dose dependently) pronounced after combined treatment of these inhibitors with TGF-β1. Similar effects were observed using 0.25 pM TGF-β1 (data not shown).

4. Discussion

It was clearly demonstrated in our experiments that all concentrations of esculetin and eicosatetraynoic acid tested significantly potentiated the inhibitory effect of TGF-β1 on [^{3}H]thymidine incorporation. The interactive polynomial models constructed from experimental data unambiguously described more than an additive effect of TGF-β1 × esculetin and TGF-β1 × eicosatetraynoic acid combinations on [^{3}H]thymidine incorporation. The expression of the overadditive effect depended both on the type and concentrations of combined agents. An exact evaluation of the interactive part of the combined effects was rather difficult at higher concentrations of the agents when the overall inhibition of [^{3}H]thymidine incorporation exceeded approximately 60%. In comparison with lower concentrations, the dose of 20 μM esculetin significantly decreased the contribution of interactive components to the total effect when esculetin was combined with all the doses of TGF-β1 used. The combinations of eicosatetraynoic acid with 0.5 pM TGF-β1 significantly lowered the contribution of model interactive components in comparison with combinations involving lower doses of TGF-β1. In spite of the quantitative differences observed, the degree of significance of the combined effects on dose–response curves supports the hypothesis of synergistic potentiation (Berenbaum, 1981) of TGF-β1 by the eicosanoid inhibitors used in our experiments.

The results of the cell cycle analysis confirmed conclusions resulting from [^{3}H]thymidine incorporation data, i.e., potentiation of antiproliferative effects of TGF-β1 by inhibitors of arachidonic acid metabolism, except some quantitative discrepancies (namely at higher concentrations of the drugs) if the effects were expressed as percentage of control. The reason for these discrepancies could be explained as follows. The number of cells in S phase need not always reflect exactly the number of cells actively synthesizing DNA. For example, the rate (dynamics) of passage of cells through S phase of the cell cycle after treatment with respective agents may be modified. Thus the proportion of cells in S and G_2/M phases of the cell cycle expresses better the antiproliferative effects of the drugs used. Moreover, the source of quantitative differences could also be related to the character of two distinct parameters, i.e., [^{3}H] thymidine incorporation data versus rather variable (binomially distributed) number of cells in S phase of the cell cycle. Relating these two effects to the control necessarily increases the variability of data — particularly in the case of the binomial variable.

Esculetin either did not affect significantly (at doses of 5 and 10 μM) or paradoxically increased the number of cells in S and G_2/M phases and lowered the number of cells in G_1 phase (at a dose of 20 μM). While after treatment of cells with TGF-β1 and eicosatetraynoic acid the lower [^{3}H]thymidine incorporation can actually reflect the lower number of cells in S phase of the cell cycle, the results obtained with esculetin could rather be due to its ability to block the cells in S phase. This situation can also result in decreased [^{3}H]thymidine incorporation. Despite the different effects of eicosanoid inhibitors on the cell cycle, their combined use with TGF-β1 resulted in the potentiation of TGF-β1 antiproliferative effects as detected by both methods used. Even if the effects on the cell cycle were observed after all tested concentrations of the drugs, the most pronounced effects were achieved after the combined treatment by TGF-β1 with lower doses of inhibitors. In the case of eicosatetraynoic acid, this is probably due to the fact that higher single doses of this drug had profound effects on the cell cycle (similarly as for [^{3}H]thymidine incorporation). The results obtained after treatment with the highest concentrations of the drugs probably reflect additional pharmacological effects. This could explain the nonlinear character of the interactions observed. The effectiveness of low doses of inhibitors allows us to suppose that these effects could be a consequence of the inhibition of enzymes catalyzing the conversion of arachidonic acid (see IC_{50} for relevant inhibitors).

The ability of esculetin and eicosatetraynoic acid to potentiate the effects of TGF-β1 on CCL64 cells, i.e., to decrease the cell number in S and G_2/M phases (and proportionally to increase the cell number in G_1 phase) together with data of [^{3}H]thymidine incorporation showed that regulatory events leading to suppression of cell division were affected. This was confirmed in experiments monitoring the effects of TGF-β1 and its combination with esculetin and eicosatetraynoic acid on cell number after 48 h of drug treatment. Moreover, the similar effects

on the cell number were observed after combined treatment of cells with TGF-β1 and MK-886 and/or indomethacin. Therefore it may be concluded that all inhibitors of arachidonic acid metabolism used in our experiments if combined with TGF-β1 led to similar effects on cell proliferation (see left part of Fig. 5). In spite of the fact that synergistic interaction was exactly demonstrated only in the experiments monitoring [^{3}H]thymidine incorporation, the significance of the results evaluating flow cytometric and cell number data indicate the multiplicative character of interaction also in these cases.

The effects observed can be explained by either (1) the lack of some (or most) arachidonic acid metabolites or (2) a certain type of misbalance in the metabolism of arachidonic acid leading to its abundance can result in modulation of TGF-β1 action. These conclusions are supported by the findings of Newman (1990), who showed that the inhibition of carcinoma and melanoma cell growth by TGF-β1 is mediated, in large part, by exogenous polyunsaturated fatty acids. Thus, the inhibition of arachidonic acid metabolism in our experiments may increase the cell pool of arachidonic acid which could act as regulator per se. The action of the drugs used, which can be independent on the metabolism of eicosanoids (Taylor et al., 1985; Anderson et al., 1989), is less possible because similar effects on cell proliferation were obtained using four structurally different compounds. Taken together, these results indicate that the inhibition of arachidonic acid metabolization and probably the concomitant enhancement of the pool of endogenous arachidonic acid can potentiate the effects of TGF-β1. TGF-β1 blocks the cell cycle in the G_1 phase by inhibiting G_1 cyclins (Geng and Weinberg, 1993; Attisano et al., 1994; Ko et al., 1995). Moreover, it was reported that polyunsaturated fatty acids may directly regulate the expression of some growth-related genes (Clarke and Jump, 1994; Dokter et al., 1994; Finstad et al., 1994). It remains to determine how the changes in endogenous arachidonic acid concentration interfere with the TGF-β1 signal transduction pathway. These experiments are in progress now.

Acknowledgements

This research was supported by grants from Grant Agency of the Czech Republic (No. 505/93/2369 and No. 524/96/1716).

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