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Short Communication

Pravastatin and Atorvastatin Combined with Aspirin Modulate Cytokine Production by Mononuclears Primed by Colon Cancer Cells

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Keywords

- Pravastatin; Atorvastatin; Mononuclears; Cancer cells; Colon carcinoma; Immune cross-talk

Abstract

Background: Aspirin and statins are drugs with a number of common activities suitable for cardiovascular diseases prevention. In addition, it seems to be well established that aspirin exerts a prophylactic effect on colorectal cancer development, whereas this quality is under debate while statins are reviewed. The aim of the present work was to examine the effect of aspirin, pravastatin and atorvastatin - each one alone or one of the statins in combination with aspirin on cell proliferation of HT-29 or RKO human colon carcinoma cells and on the immune cross talk between peripheral blood mononuclear cells (PBMC) and the cancer cells.

Methods: PBMC stimulated for cytokine production by HT-29 and RKO cells were incubated with aspirin, pravastatin, atorvastatin alone, or jointly with aspirin, and the release of TNFα, IL-1β, IL-6, IL-2, IFNγ, IL-10 and IL-1ra was detected.

Results: While aspirin had no effect on IL-1β secretion, the enhancement caused by pravastatin and atorvastatin was reduced upon aspirin addition. The production of TNFα and IFNγ was stimulated by aspirin it was inhibited by atorvastatin and was not affected by pravastatin. IL-10 secretion was markedly inhibited by atorvastatin, slightly reduced by pravastatin and aspirin and was supplementary reduced when statins and aspirin were added together. The production of IL-1ra induced by RKO but not HT-29 cells was stimulated by pravastatin or aspirin and by the combination of atorvastatin with aspirin.

Conclusions: The results indicate that co-incubation of one of the statins with aspirin and PBMC stimulated by colon cancer cells ensues in modulation of the immune dialogue between mononuclears and colon carcinoma cells. These observations may further clarify the way pravastatin, atorvastatin and aspirin affect colon cancer development acting alone or administered jointly.

INTRODUCTION

Evaluating the longevity and worldwide use of drugs, aspirin deserves to be at the top of the list. Notably, the more its use continues, the more properties beneficial to human health have been attributed to this remarkable drug. Known since long for his analgesic and antipyretic effects, additional attributes such as antithrombotic, anti-inflammatory, and even anti-cancer activities have been recognized and have been the subject of a great number of scientific reports. Besides its anti-inflammatory activities, it has been asserted that aspirin is an immunomodulatory agent capable to promote the function of immune cells such as macrophages, T and B lymphocytes and polymorphonuclear cells [1-3]. An intriguing review of the history of aspirin beginning with its discovery till clarifying the molecular mechanism of its therapeutic effects has been reported by Miner and Hoffhines [4]. The antithrombotic activity of aspirin due to inhibition of platelet aggregation by acetylation of the platelet cyclooxygenase was applied as an important prevention against life threatening vascular events, such as ischemic heart disease, myocardial infarction and occlusive stroke [5,6]. However, coming to understanding that one of the main culprits for development of vascular obstruction and atherosclerosis is elevated cholesterol level, it became evident that antiplatelet therapy by itself cannot be sufficient for prevention of vascular events. Therefore, scientists became involved in exploring cholesterol synthesis, its metabolism, the way to impede its production and reached the conclusion that inhibition of 3-hydro-3-methl/glutaryl coenzyme A (HMG-CoA) reductase lowers cholesterol level. Consequently Akira Endo synthesized a number of compounds that gradually led to development of statins used today by millions of hypercholesterolemic patients [7]. With time it became clear that statins possess pleiotropic properties and exert anti-inflammatory activities on the vascular walls in part by their ability to modulate the production of anti-inflammatory cytokines [8,9], and by converting M1- to M2 macrophage phenotype [10]. It is of interest that the impact of hydrophilic statins on cytokine production by immune cells and phagocytic capacity differs from that of the hydrophobic ones [11,12]. Following studies demonstrating close relation between chronic inflammation, innate immunity and cancer development [13],

the anti-inflammatory capacities of aspirin rapidly introduced the drug into the anticancer amentarium. Reports have shown that daily doses of 75-100mg. aspirin may reduce incidence, metastasis and mortality of colorectal cancer [14-16]. One way to explain the efficacy of aspirin to reduce colorectal cancer risk is its ability to suppress COX-2 production that is overexpressed in this and other malignant conditions [17-19]. Since aspirin and statins share immunomodulatory and antithrombotic activities and therefore they are administered often in combination, the question arises if statins are also chemopreventive agents. As for their beneficial activity in prevention of colorectal cancer, the researchers' opinion remains disputable. Based on meta-analyses of great number of studies, one gains the impression that the reduction of cancer risk in individuals under long treatment with statins is quite modest [20-22]. Bergman et al. [23], have reported that simvastatin (hydrophobic) but not pravastatin (hydrophilic) is able to inhibit the proliferation of cells from two human colon cancer lines. It has been shown that while aspirin, atorvastatin and pravastatin may affect cytokine production by human peripheral blood mononuclear cells (PBMC), aspirin applied together with statins acts as a better immune promoter [24]. The present study was aimed to examine the capacity for cytokine production by PBMC co-incubated with HT-29 or RKO colon cancer cells under the effect of aspirin, atorvastatin and pravastatin, each one alone and in alliance. The results may serve as an indicator if the association between aspirin and statins is a better implement for colon cancer risk reduction.

MATERIALS AND METHODS

Statins and aspirin preparation

Atorvastatin and pravastatin were kindly provided by Teva, Pharmaceutical Industries Ltd., API Division, Petah-Tiqva, Israel. Atorvastatin calcium was dissolved in dimethyl sulphoxide (DMSO, Sigma, Israel) and pravastatin in phosphate buffered saline (PBS). A stock solution of 5mM of each statin was prepared with the appropriate diluent, and further dilutions were produced in medium. The statins were added to the cell cultures at a final concentration of 50μM. Aspirin (Acetylsalycilic acid, Sigma, Israel) was dissolved in absolute ethanol at 100 mg/ml. Dilutions were prepared in RPMI-1640 medium containing 1% penicillin, streptomycin and nystatin, 10% fetal bovine serum (FBS, Biological Industries, Beith Haemek, Israel) and was designated as complete medium (CM) and the pH was adjusted to 7.0 using 1N NaOH. Further dilutions were carried out in CM. Aspirin was added to the cultures at a final concentration of 10μg/ml. Control cultures were incubated either with medium or with ethanol or DMSO at final concentrations of 0.2% and 1% corresponding to the concentrations added with the statins.

Peripheral blood mononuclear cells

Buffy coats obtained from donors' blood were purchased from Magen David Adom, Blood Services Center in Israel after signing an informed consent containing a written agreement that blood components not suitable for therapeutic needs may be used for medical research. PBMC were separated by Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation. The cells were washed twice in phosphate buffered saline (PBS) and suspended in CM.

Colon cancer cell lines

HT-29 and RKO human colon cancer cell lines were obtained from American Type Cultural Collection, Rockville, MD. The cells were maintained in CM containing Mc-Coy's 5A medium and Dulbecco modified eagle medium (DMEM) respectively, supplemented with 10% FBS, 2mM L-glutamine and antibiotics (penicillin, streptomycin and nystatin-Biological Industries Co, Beith-Haemek, Israel). The cells were grown in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO2. Since the cells from both lines adhere to the flasks they were removed using trypsin/EDTA solution (Biological Industries Co, Beith-Haemek, Israel.

Effect of statins and aspirin on cell proliferation

The effect of statins and aspirin on cell proliferation was determined using XTT proliferation assay kit (Biological Industries, Beith Haemek, Israel). Briefly, 0.1 ml aliquots of HT-29 or RKO colon cancer cells (105/ml of CM) obtained after trypsinization and suspended in appropriate CM were added to each one of 96 well plates and incubated for 24 hrs in the absence or presence of pravastatin or atorvastatin (50μM), with aspirin (10μg/ml) or with a combination of one of the statins and aspirin at concentrations as indicated. At the end of the incubation period the cells were stained according to the manufacturer's instructions. The plates were incubated for 2-4 hrs at 37°C in a humidified incubator containing 5% CO2 and the absorbance was measured at 450 nm using ELISA reader.

Effect of aspirin and statins on cytokine production

HT-29 or RKO cells were suspended in the appropriated CM at 4x105/ml. The cells were seeded in 24 well plates by addition of 0.5ml of cell suspension to each well. The plates were incubated for 60 min at 37°C in a humidified atmosphere containing 5% CO_2 before adding 0.5ml aliquots of PBMC suspended in CM (4x106/ml) to each well. Cells were incubated without or with pravastatin or atorvastatin (50μM), with aspirin (10μg/ml) or with a combination of one of the statins and aspirin at concentrations as indicated. All drugs were added at the onset of cultures. Cultures were maintained for 24 hrs at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, the culture media were collected, the cells were removed by centrifugation at 200g for 10 min and the supernatants were kept at -70°C until assayed for cytokine content.

Cytokine content in the supernatants

The concentration of cytokines in the supernatants was tested using ELISA kits specific for human cytokines (Biosource International, Camarillo, CA) as detailed in the guide-line provided by the manufacturer. The detection level of all cytokines was 30pg/ml.

Statistical analysis

Paired t-test was used to compare between the level of cytokines produced following incubation with the drugs and that found in control cultures. In addition, paired t-test was used to compare between cells incubated with one drug and that incubated with a combination of a statin and aspirin. Probability values of $p<0.05$ were considered as significant. The results are

expressed as mean ± SEM of six experiments using different blood donors.

RESULTS

Effect of statins and aspirin on cell proliferation

Twenty four hours of incubation of either HT-29 or RKO colon cancer cells with either aspirin or pravastatin or in combination had no effect on cell proliferation as examined by XTT assay. However, incubation of HT-29 or RKO cells with atorvastatin or atorvastatin with aspirin caused 42% and 31% inhibition of HT-29 cell proliferation respectively ($p<0.005$), and 28% inhibition ($p<0.001$) of RKO cell proliferation. The reduction of both malignant cell proliferation caused by incubation with both atorvastatin and aspirin did not differ significantly from that induced by atorvastatin alone (Table 1).

Cytokine secretion by colon cancer cells

No detectable concentrations of cytokines tested could be found in supernatants obtained from 24 hrs cultures of either HT-29 or RKO cells incubated at the above- mentioned conditions without or with one of the drugs alone or in combination (aspirin with one of the statins) at concentrations as indicated.

Effect of statins and aspirin on cytokine production

TNFα: While pravastatin had no effect on TNFα secretion by PBMC induced by either HT-29 or RKO colon cancer cells, atorvastatin caused 49% and 73.5% inhibition ($p<0.001$) of this cytokine production, respectively. Aspirin added alone to HT-29 or RKO cells caused 12.6% and 9.5% stimulation of TNFα production, respectively ($p<0.01$). Similar stimulation of TNFα secretion was found when aspirin was added together with pravastatin. Addition of both aspirin and atorvastatin slightly attenuated the inhibitory effect caused by atorvastatin alone (Figure 1).

IL-1β: The production IL-1β by PBMC induced by HT-29 colon cancer cells was enhanced upon incubation with atorvastatin and pravastatin by 24% and 18% respectively ($p<0.01$), whereas that induced by RKO cells was increased by 33% following addition of atorvastatin ($p<0.02$). Aspirin added alone had no effect on IL-1β secretion induced by both cell lines. However, when aspirin was added together with either atorvastatin or pravastatin, the enhanced production of IL-1β caused by the statins was significantly attenuated ($p<0.05$, Figure 2).

IL-2 and IL-6: The production of IL-2 or IL-6 by PBMC stimulated by either HT-29 or RKO colon cancer cells was not significantly affected by 24 hrs of incubation with atorvastatin, pravastatin of aspirin added separately or in a combination of one of the statins with aspirin (Table 2).

Table 1: Effect of statins and aspirin on cell proliferation.

Drug added	HT-29		RKO	
	OD at 450nm	P value	OD at 450nm	P value
0	1924±75		1058±24	
Pravastatin, 50μM	2016±75	NS	1062±10	NS
Atorvastatin, 50μM	1109±170	0.0047	768±30	0.0002
Aspirin, 10μg/ml	2064±64	NS	1082±38	NS
Aspirin + Pravastatin	1880±49	NS	1042±18	NS
Aspirin +Atorvastatin	1323±46	0.0005	764±30	0.0002

HT-29 or RKO colon cancer cells (10^5/ml of CM) were incubated for 24 hrs in the absence (0) or presence of pravastatin, atorvastatin or aspirin at concentrations as indicated or with a combination of aspirin with one of the statins at the same concentrations. At the end of the incubation period XTT test was carried on according to the manufacturer's instructions. The plates were incubated for 2 hrs at 37°C in a humidified incubator containing 5% CO_2 and the absorbance was measured at 450nm using ELISA reader. The results are expressed as the Mean ± SEM of 4 replicates. P value represents statistically significant difference from cells incubated without any drug (0), whereas NS not statistically significant different from cells incubated without any drug.

Table 2: Effect of statins and aspirin on IL-2 and IL-6 production by PBMC.

	IL-2, ng/ml		IL-6, ng/ml	
	HT-29	RKO	HT-29	RKO
0	4.41±0.27	3.44±0.12	25.5±0.6	25.3±0.7
Atorvastatin	4.25±0.38	4.21±0.58	25.0±1.0	24.8±1.1
Pravastatin	4.34±0.24	3.37±0.12	24.3±0.8	24.8±0.9
Aspirin	4.63±0.33	3.45±0.07	23.7±0.7	24.3±0.5
Asp+atorva	4.41±0.42	4.11±0.51	24.6±1.0	25.6±0.8
Asp+prava	4.75±0.42	3.46±0.10	24.0±0.3	24.6±0.5

$2x10^6$ PBMC suspended in 1 ml of CM were incubated for 24 hrs with either HT-29 or RKO colon cancer cells in the absence (0) or presence of each one of the followings: pravastatin, atorvastatin (50μM) or aspirin 10μg/ml and with a combination of aspirin and one of the statins. At the end of the incubation period supernatants were collected and tested for cytokines using ELISA kits. The results are expressed as Mean ± SEM of 6 experiments.

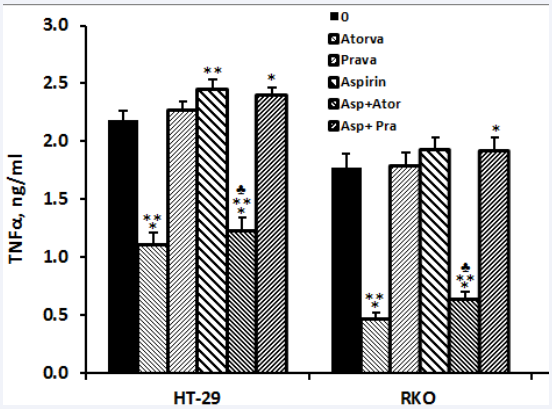

Figure 1 Effect of aspirin and statins on TNFα production
2x10^6 PBMC suspended in 1 ml of CM were incubated for 24 hrs with either HT-29 or RKO colon cancer cells in the absence (0) or presence of one of the followings: pravastatin, atorvastatin (50μM) or aspirin 10μg/ml alone, or with a combination of aspirin and one of the statins. At the end of the incubation period supernatants were collected and tested for TNFα using ELISA kits. The results are expressed as Mean ± SEM of 6 experiments. Asterisks represent statistically significant difference from cells incubated without the drugs (0) (*p<0.05; **p<0.01; ***p<0.001). represents statistically significant difference from cells incubated with atorvastatin only.

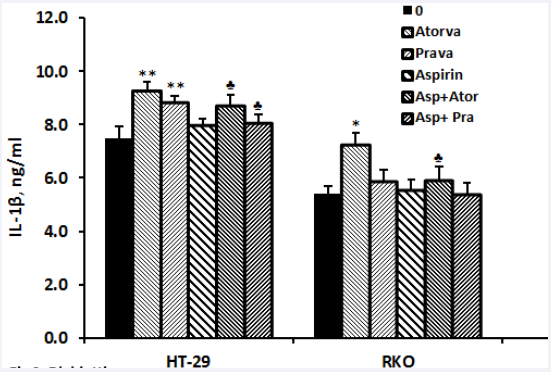

Figure 2 Effect of aspirin and statins on IL-1β secretion
2x10^6 PBMC suspended in 1 ml of CM were incubated for 24 hrs with either HT-29 or RKO colon cancer cells in the absence (0) or presence of one of the followings: pravastatin or atorvastatin (50μM), aspirin 10μg/ml alone, or with a combination of aspirin and one of the statins. At the end of the incubation period supernatants were collected and tested for IL-1β using ELISA kits. The results are expressed as Mean ± SEM of 6 experiments. Asterisks represent statistically significant difference from cells incubated without drugs (0) (*p<0.05; **p<0.01). represents statistically significant difference from cells incubated with statin.

IFNγ: Atorvastatin inhibited the secretion of IFNγ by PBMC prompted by either HT-29 or RKO cells by 42% and 27% respectively (p<0.05), whereas incubation with aspirin caused 27% and 20% enhancement in IFNγ production, respectively (p<0.05). The reduced production of IFNγ induced by both cell lines upon incubation with atorvastatin was not affected significantly when aspirin was added. Incubation with pravastatin had no effect on this cytokine secretion. In addition the stimulatory effect caused by aspirin on IFNγ production was also not affected by pravastatin (Figure 3).

IL-10: The secretion of IL-10 by PBMC induced by HT-29 cells following incubation with atorvastatin, pravastatin and aspirin was inhibited by 80% (p<0.001), 15% (p<0.05) and 19% (p<0.01) respectively, and that induced by RKO cells was lowered by 85% (p<0.001), 13% (p<0.02) and 18% (p=0.135), respectively. IL-10 secretion induced by HT-29 was further lowered by 85% (p=0.005) and 36% (p<0.05) when the cells were incubated with atorvastatin or pravastatin together with aspirin, as compared to cells incubated with each one of the statins alone. The production of IL-10 induced by RKO cells was further reduced by 36% (p<0.05) upon incubation with pravastatin and aspirin jointly, as compared to cells incubated with pravastatin alone (Figure 4).

IL-1ra: The production of IL-1ra by PBMC prompted by HT-29 cells was not affected when the cultures were incubated with statins or aspirin alone or in combination. IL-1ra secretion by PBMC stimulated by RKO cells was not affected by atorvastatin alone, but was enhanced by 31% and 16% (p<0.01) when the cells were incubated with pravastatin or aspirin, respectively. Combination of atorvastatin and aspirin caused 29% enhancement in IL-1ra secretion (p<0.02), results significantly different from that produced by cells incubated with each one of the drugs alone (p<0.05, Figure 5).

DISCUSSION

The factors capable to modulate the cross-talk between immune and cancer cells play important role in cancer development. It seems to be well established that administration of aspirin, even at low doses, exerts a prophylactic effect by reducing the risk of colon cancer. Previous studies have shown that aspirin may affect the cross-talk between mononuclear and colon cancer cells from two human lines [25]. As for statins, the opinions about their activity in preventing tumorigenesis are uncertain, although in animal models the results have been

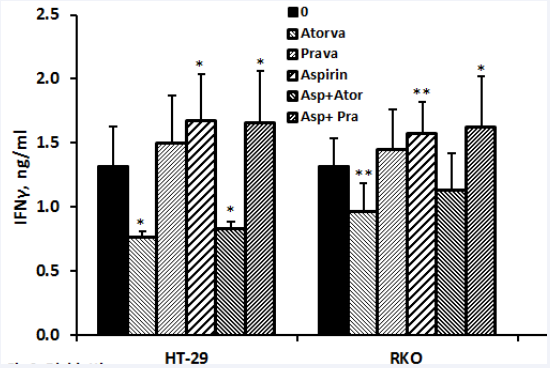

Figure 3 Effect of aspirin and statins on IFNγ secretion
2x10^6 PBMC suspended in 1 ml of CM were incubated for 24 hrs with either HT-29 or RKO colon cancer cells in the absence (0) or presence of one of the followings: pravastatin or atorvastatin (50μM), aspirin 10μg/ml alone, or with a combination of aspirin and one of the statins. At the end of the incubation period supernatants were collected and tested for IFNγ using ELISA kits. The results are expressed as the Mean ± SEM of 6 experiments. Asterisks represent statistically significant difference from cells incubated without drugs (0) (*p<0.05; **p<0.001).

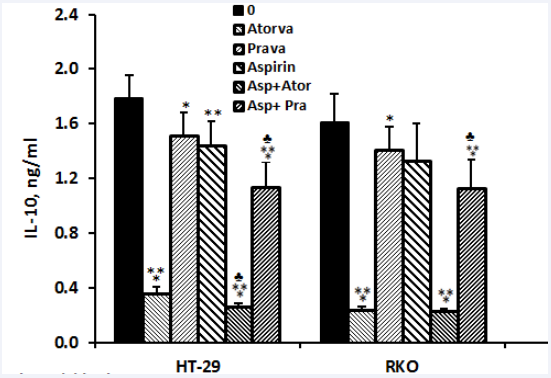

Figure 4 Effect of aspirin and statins on IL-10 secretion
$2x10^6$ PBMC suspended in 1 ml of CM were incubated for 24 hrs with either HT-29 or RKO colon cancer cells in the absence (0) or presence of one of the followings: pravastatin or atorvastatin (50μM), aspirin 10μg/ml alone, or with a combination of aspirin and one of the statins. At the end of the incubation period supernatants were collected and tested for IL-10 using ELISA kits. The results are expressed as Mean ± SEM of 6 experiments. Asterisks represent statistically significant difference from cells incubated without drugs (0) (*p<0.05; **p<0.01;**p<0.001). represents statistically significant difference from cells incubated with statins.

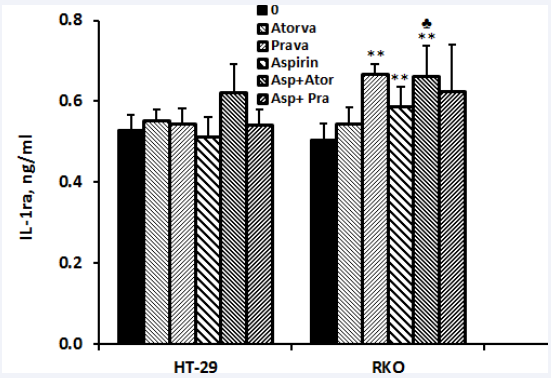

Figure 5 Effect of aspirin and statins on IL-1ra secretion
$2x10^6$ PBMC suspended in 1 ml of CM were incubated for 24 hrs with either HT-29 or RKO colon cancer cells in the absence (0) or presence of one of the followings: pravastatin or atorvastatin (50μM), aspirin 10μg/ml alone, or with a combination of aspirin and one of the statins. At the end of the incubation period supernatants were collected and tested for IL-1ra using ELISA kits. The results are expressed as Mean ± SEM of 6 experiments. Asterisks represent statistically significant difference from cells incubated without drugs (0) (**p<0.01). represents statistically significant difference from cells incubated with atorvastatin or aspirin.

fairly convincing [26]. Research to achieve chemoprevention of colon cancer by jointly administration of non-steroidal anti-inflammatory drugs with agents capable to affect the growth rate of colon carcinoma cells showed positive outcome at least in vitro [27]. Inhibition of cancerous prostate cells' growth by clinical doses of aspirin and statins has been reported by Murtola et al [28]. These observations prompted us to examine the effect of two drugs frequently administered together i.e. aspirin and one of the statins- atorvastatin (hydrophobic) and pravastatin (hydrophilic) on cell proliferation of two brands of colon carcinoma cells and the capacity for cytokine production by PBMC stimulated by cancer cells. Analysis of the results showed that applied separately only aspirin and atorvastatin were able to inhibit cancer cell proliferation. Jointly administration of aspirin with atorvastatin increased the inhibitory effect of this statin on that cell activity. The three drugs, alone or in combination (aspirin and one of the statins) did not affect the production of IL-6 and IL-2 by PBMC co-incubated with colon cancer cells from the two lines. It has been shown in a previous study that pravastatin and aspirin were not capable to affect the secretion of IL-6 by PBMC, whereas that of IL-2 was repressed by atorvastatin and was further inhibited by addition of aspirin. In the same work the generation of IL-1ra was mildly enhanced by pravastatin and aspirin separately and by their combination [24]. Increased expression of proinflammatory cytokines following treatment of macrophages with statins has been reported in other studies, although variations have been linked with the type of the drugs [29]. Using co-cultures of human vascular smooth muscle cells and PBMC, Loppnow et al. [30], have found that atorvastatin and pravastatin reduced IL-6 production in a dose-dependent manner and it was completely inhibited following incubation of statins and aspirin applied in combination. In our hands, the production of TNFα and IFNγ behaved comparably in co-cultures of PBMC with HT-29 cancer cells following incubation with aspirin and statins. While pravastatin did not affect their production alone, the drug administered jointly with aspirin induced enhanced secretion. However, since that increase was not different from the one exerted by aspirin alone, it is conceivable to assume that it was due to aspirin. At comparable co-cultures, aspirin and atorvastatin separately or jointly acted similarly, but in that case aspirin stimulated TNFα and IFNγ production. Attenuation of LPS induced TNFα production by macrophages has been observed by other researchers upon the effect of atorvastatin [31]. In the present work IL-1β secretion by PBMC stimulated by HT-29 cells was promoted by both statins and it was less elevated when the statins were linked with aspirin. It is notable that at the same laboratory set-up the production of a few of the pro-inflammatory cytokines by PBMC stimulated by RKO cancer cells differed from that promoted by HT-29 cells. The generation of the anti-inflammatory cytokine IL-10 was inhibited by all three drugs alone and even further reduced when applied in combination, independently on the mode of PBMC stimulation either by HT-29 or RKO cancer cells. On the other hand, aspirin and statins, alone or in combination had no effect on the production of the anti-inflammatory cytokine IL-1ra by PBMC stirred with HT-29 cells, while its generation by PBMC stimulated by RKO cells was enhanced. These results point out to the fact that the interaction among immune, cancer cells and drugs, at least at the present laboratory set up, is cell-and drug dependent. The immune dialogue between mononuclears and carcinoma cells that proceeds in the chronic inflammatory microenvironment in malignant tumors is one of the key players in cancer development and is connected with release of inflammatory cytokines and oncogenic mediators [32]. Hypoxia and cell death have been reported to affect the cross-talk between macrophages and cancer cells [33]. Working with murine tumor cell lines Beury et al. [34], have observed that incubation of tumor cells with macrophages increased the production of macrophage IL-6 and decreased that of TNFα. Mediators capable to affect macrophage-tumor cell

immune relationship play an important role in tumorigenesis [35]. It has been found that addition of hyaluronan reverses the inhibited production of reactive oxygen intermediates released by monocytes pre-incubated with tumor cells [36]. Efforts to combine low doses of non-steroidal anti-inflammatory drugs with other agents including statins for colon cancer chemoprevention have been forwarded previously [27,37,38].

In conclusion, the presented findings contribute to apprehend the role of aspirin applied alone or jointly with one of two statins, one pravastatin (hydrophilic) and the other one atorvastatin (hydrophobic) on the immune cross-talk between human peripheral blood mononuclears and human colon carcinoma cells from two different lines. The results demonstrate that the immunomodulation exerted by the three drugs is dependent of the colon carcinoma cell type, as well as on the way they are applied and underline their potential influence on the therapeutic outcome in colon cancer patients.

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