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Arachidonic Acid Metabolism in Growth Control of A549 Human Lung Adenocarcinoma Cells

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Abstract—The role of individual eicosanoids of the arachidonic acid (AA) cascade in the growth control of A549 human lung adenocarcinoma cells has been studied. Cyclooxygenase and lipoxygenase metabolites of [^{14}C]AA incorporated were actively synthesized in the cultures of tumor cells with full confluence unaccomplished. In such cultures inhibitors of AA metabolism (indomethacin and esculetin) and also a lipoxygenase metabolite of AA, 15-hydroxyeicosatetraenoic acid (15-HETE), significantly suppressed the incorporation of [^{3}H]thymidine and biosynthesis of prostaglandin E_2 (PGE_2). Other lipoxygenase metabolites of AA (5-HETE and 12-HETE) had no effect on these parameters. The basic fibroblast growth factor (bFGF) had practically no affect on the growth of A549 cells and the PGE_2 production in cultures with 5% fetal calf serum (FCS); however, in the presence of 0.5% FCS this factor significantly increased the number of tumor cells. The growth-stimulating effect of bFGF was completely abolished by a cyclooxygenase inhibitor indomethacin. The data suggest a key role of PGE_2 in the growth control of A549 cells with an active synthesis of cyclooxygenase and lipoxygenase metabolites of AA, its importance in realization of the mitogenic effect of bFGF, and specific features of 15-HETE as a down-regulator of the PGE_2-dependent proliferation.

Key words: arachidonic acid metabolism, human tumor cells, cell proliferation, basic fibroblast growth factor

Many data in the literature suggest a great importance of eicosanoids of the arachidonic acid (AA) cascade in pathogenesis of malignancies. In this aspect the best studied are cyclooxygenase metabolites of AA, prostanoids, which markedly modulate the initiation and promotion of inducible tumors in animals [1], induce and mediate the expression of some oncogenes [2, 3], display a strong immunosuppressive effect [4], and induce angiogenesis [5]. However, individual oncogenes, chemical carcinogens, promoters, and many other factors which play an active role during carcinogenesis are strong inducers of cyclooxygenase and, as a result, of prostanoid biosynthesis [6-8]. The role of eicosanoids in processes of tumor growth and metastasizing is rather complicated and not clear because they display features of both negative and positive modulators. In particular, many prostanoids can induce differentiation [9] and suppress the growth of tumor cells along with the antiproliferative [10] or cytotoxic effect [11]. However, the basic cyclooxygenase metabolite of AA, prostaglandin E_2 (PGE_2) is a growth-limiting factor for many tumor cells [12, 13]. Similar features were recently found also in lipoxygenase metabolites of AA, such as 5- and 12-hydroxyeicosatetraenoic acids (5- and 12-HETE) [14-17]. Therefore, it is exclusively important to compare the roles of individual eicosanoids in the growth control of various normal and tumor cells with different profiles of AA metabolites produced and also their interactions with growth factors, first of all, with the multifunctional basic fibroblast growth factor (bFGF), which is a key link in the transduction of the signal system of AA metabolism activation in cells. We have shown earlier that the clonal proliferation of rat bone marrow stroma fibroblasts with the predominant synthesis of PGE_2 and the mitogenic effect of bFGF directly correlate with the production of this eicosanoid [18]. The purpose of the present work was to study the interrelation of the AA metabolism with the proliferation and with the growth-modulating effect of bFGF in human lung cancer line A549.

MATERIALS AND METHODS

Vessels and reagents. Plastic flasks for cell cultures were obtained from Corning Costar (USA). Fetal calf serum (FCS) and the DMEM medium were purchased

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from Life Technologies (Great Britain), the RPMI-1640 medium, metabolites of AA (5-, 12-, and 15-HETE), inhibitors of AA metabolism (indomethacin and esculetin), and bFGF were purchased from Sigma (USA), [1-^{14}C]AA with the specific activity of 54 mCi/mmol and the labeled standards were obtained from Amersham (Great Britain). [Methyl-^{3}H]thymidine with the specific activity of 50 Ci/mmol was prepared in the Institute of Molecular Genetics, Russian Academy of Sciences. Other reagents were of domestic production, of chemical and special chemical purity.

Cell cultures. A549 human lung adenocarcinoma cells (American Type Culture Collection, USA) were grown at 37°C in a humidified atmosphere with 5% CO_2 in the DMEM and RPMI-1640 media at the ratio of 1 : 1 (the total volume was 5 ml) supplemented with FCS (5%), penicillin (100 units/ml), and streptomycin (100 μg/ml). During the culture growth the medium was usually changed every day. Depending on the experiment purpose, the starting density was varied from 0.08 to $2.00 \cdot 10^5$ cells/ml. The factors under study were introduced on the second or third day of the culture growth. The final concentration of bFGF in the culture was 20, 40, and 80 ng/ml, the concentration of 5-, 12-, and 15-HETE was 10 ng/ml, the concentration of indomethacin was 10^{-5} and 10^{-6} M, and that of esculetin was 10^{-5} M. The effects of the factors on the cell proliferation and biosynthesis of eicosanoids were assessed 72-96 h after starting the culture.

Assessment of cell proliferation. The A549 cell proliferation was evaluated by the cell growth density in culture and by the incorporation of [^{3}H]thymidine into DNA. [^{3}H]Thymidine (1 μCi/ml) was introduced at different times of the culture growth by pulses for 1 h, then the excess label was removed by a threefold washing of the cells with the medium without FCS. For counting in a Goryaev's chamber and for scintillation counting, adhesive cells were taken with 0.25% EDTA. The radioactivity (cpm) was determined with a Wallac 1219 liquid scintillation counter (LKB, Sweden) [14].

Study of [^{14}C]AA metabolism. The biosynthesis of eicosanoids from [^{14}C]AA incorporated into the cells was studied on the first to third and on the second to fourth days of culture growth. The cells were incubated with the labeled precursor (0.1 μCi/ml) at 37°C for 24 h and then the [^{14}C]AA incorporated was removed by threefold washing with the medium without FCS. The effects of bFGF, of inhibitors of the AA metabolism, and of AA metabolites on the eicosanoid biosynthesis were assessed on the 24-h incubation with the factors after the label incorporation. The eicosanoids synthesized were extracted from the medium twice with three volumes of ethyl acetate, and the solvent was subsequently evaporated under a flow of nitrogen. The metabolites of [^{14}C]AA were separated by TLC (Kieselgel 60 plates, Merck, Germany) using the organic phase of the solvent system of ethyl acetate–isooctane–CH_3COOH–H_2O (11 : 5 : 2 : 10 v/v) and the standards labeled [18]. The total ^{14}C-radioactivity of the specimens was determined with a Wallac 1219 counter. The profile of eicosanoids synthesized was analyzed on autoradiochromatograms on an X-Omat XAR-5 X-ray film (Kodak, USA) using a KS 3 densiskan (Kipp and Zonen, Holland).

Statistical processing of results. The average values of the A459 cell proliferation and of the eicosanoid biosynthesis and the standard error were determined for three or four cultures in three independent experiments. The significance of the findings was evaluated with Student's *t*-test.

RESULTS

Incorporation of [^{3}H]thymidine into DNA of intact A549 cells. The standard conditions of A549 cell culture in medium with 5% FCS usually led to 50-80% confluence by the fourth day of growth. The monolayer was completed by the fourth day of culture growth when the starting density was 10^5 cells/ml. The [^{3}H]thymidine incorporation in intact tumor cell cultures achieved complete confluence by this time had maximum (up to 350 cpm per 10^3 adhesive cells) on the first day of the growth, with a significant decrease in the radioactivity during the subsequent three days (Fig. 1). The different [^{3}H]thymidine incorporation into DNA during the culture growth was concordant with a rapid duplication of the cell number from the first to the second day and with more than a twofold decrease in their growth rate during the second to fourth days of culture growth.

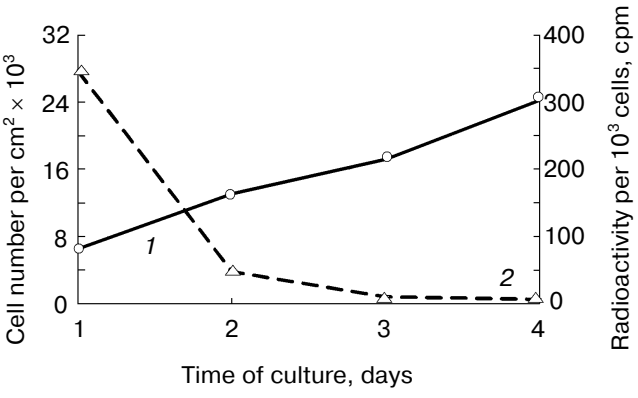

Fig. 1. Growth of A549 cells (*1*) and incorporation into them of [^{3}H]thymidine (*2*) on the different days of culture in the medium with 5% FCS. The starting density of the culture was 10^5 cells/ml. [^{3}H]Thymidine was introduced 24, 48, 72, and 96 h after the beginning the culture by 1-h pulses at the concentration of 1 μCi/ml. The radioactivity of the cells was measured as described in "Materials and Methods".

The [^{3}H]thymidine incorporation into the cells also markedly depended on their number in the flask. At the low starting density ((0.8-3.2)·10^4 tumor cells per ml of the medium) on the fourth day of the culture growth the label was 20-50 cpm per 10^3 adhesive cells. At the higher starting density ((0.64-1.0)·10^5 tumor cells per ml) the [^{3}H]thymidine incorporation was decreased to 5-6 cpm per 10^3 adhesive cells.

Spontaneous biosynthesis of eicosanoids from [^{14}C]AA incorporated in control cultures of A549 cells. Unlike normal rat bone marrow stroma fibroblasts which mainly synthesize PGE_2 [18], A549 cells actively synthesized from [^{14}C]AA incorporated not only cyclooxygenase but also lipoxygenase metabolites. The label incorporation into $PGF_{2\alpha}$, PGE_2, 5-HETE, and also into 12- and 15-HETE in the control cultures under standard conditions (5% FCS, fourth day of growth, 80% confluence) was 22.22 ± 3.17, 26.03 ± 3.81, 42.54 ± 6.35, and 21.59 ± 3.08 cpm per 10^4 cells, respectively. The profiles of eicosanoids synthesized in A549 cells determined by densitometry of radioautographs of thin layer chromatograms allowed us to assess the relative conversion of the labeled precursor into different metabolites. This parameter markedly depended on conditions and on time of the tumor cell growth. Thus, in the control cultures of A549 cells on the fourth day of growth in medium unchanged before the introduction of [^{14}C]AA (48 h after beginning the culture) the $PGF_{2\alpha}$, PGE_2, 5-HETE, and 12- and 15-HETE fractions in the profile were 3.5 ± 0.5, 4.1 ± 0.6, 6.7 ± 1.0, and 3.4 ± 0.5%, respectively. However, the medium replacement before the label introduction significantly changed the profile of metabolites synthesized in the cells, with an insignificant increase in the fraction of PGE_2 and with a complete suppression of production of 12- and 15-HETE ($PGF_{2\alpha}$, PGE_2, 5-HETE, and of 12- and 15-HETE fractions were 3.4 ± 0.5, 4.5 ± 0.7, 5.3 ± 0.9, and 0%, respectively). However, on the third day of the tumor cell growth (introduction of [^{14}C]AA 24 h after beginning the culture) the conversion of the labeled precursor into eicosanoids was more pronounced, especially into cyclooxygenase metabolites ($PGF_{2\alpha}$, PGE_2, 5-HETE, and 12- and 15-HETE fractions were 6.9 ± 1.1, 7.3 ± 1.2, 8.6 ± 1.3, 4.0 ± 0.7%, respectively) (Fig. 2). Similar changes in the biosynthesis of the [^{14}C]AA metabolites were found at decreased number of the plated cells. Note, that on the fourth day of the growth the total synthesis of eicosanoids in the cultures with a low density of the tumor cell growth was more than fourfold

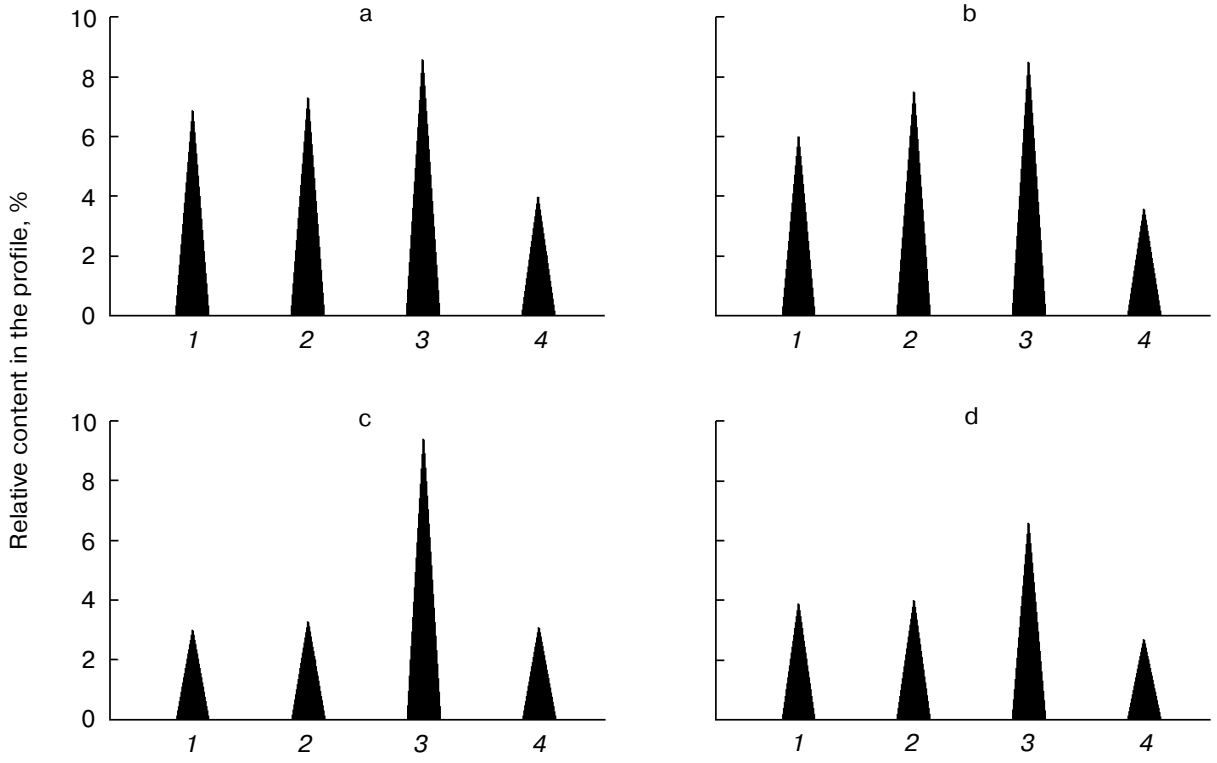

Fig. 2. Synthesis of $PGF_{2\alpha}$ (*1*), PGE_2 (*2*), 5-HETE (*3*), 12- and 15-HETE (*4*) from [^{14}C]AA incorporated in control cultures of A549 cells (a) with 5% FCS and on addition of bFGF (b), 15-HETE (c), and esculetin (d). [1-^{14}C]AA was introduced for 24 h on the second day of culture, then the cell were washed from the excess of the labeled precursor and incubated with the preparations under study for the subsequent 24 h. Results of counting of densitograms of radioautographs of thin layer chromatograms are presented.

higher than in the cultures which had the complete confluence accomplished.

Effects of inhibitors of AA metabolism and of AA metabolites on incorporation of [^{3}H]thymidine into DNA of A549 cells. Inhibitors of AA metabolism effectively suppressed the [^{3}H]thymidine incorporation into DNA when they were present in the culture from the second to the third day of growth, i.e., during the most intense biosynthesis of eicosanoids in A549 cells. Indomethacin, a selective inhibitor of cyclooxygenase, suppressed the growth adenocarcinoma cells more markedly, than esculetin. Thus, in the control the [^{3}H]thymidine incorporation was 40.50 ± 9.06 cpm per 10^3 adhesive cells and in the presence of indomethacin this parameter significantly decreased to 7.29 ± 0.23 cpm per 10^3 adhesive cells (−82% compared to the control, $p < 0.01$). The label incorporation into DNA under the influence of esculetin inhibition of both pathways was 20.09 ± 0.83 cpm per 10^3 adhesive cells (−50% compared to the control, $p < 0.05$).

Note, that 5- and 12-HETE, which are growth-stimulating factors for some tumor cells, had no effect on the [^{3}H]thymidine incorporation into DNA of A549 cells. These parameters for 5- and 12-HETE were 39.34 ± 1.81 and 38.56 ± 1.39 cpm per 10^3 adhesive cells, respectively (−3 and −5%, respectively, compared to the control) (Fig. 3). Of the lipoxygenase AA metabolites under study, only 15-HETE modulated the cell growth. The presence of this eicosanoid in the cultures decreased the incorporation of the labeled thymidine to 18.09 ± 1.29 cpm per 10^3 adhesive cells (−55% compared to the control, $p < 0.05$).

At the same time, indomethacin, esculetin, and 15-HETE markedly suppressed also the biosynthesis of PGE_2 which is the main cyclooxygenase metabolite of AA (Figs. 2 and 3). Indomethacin was the most effective in suppression of this biosynthesis: at the concentration of 10^{-5} M, it practically completely inhibited the production of PGE_2. Inhibition of the biosynthesis of PGE_2 with esculetin was near 45%, compared to control, and only 15-HETE among the lipoxygenase metabolites of AA affected the production of this prostanoid, suppressing it by 55%.

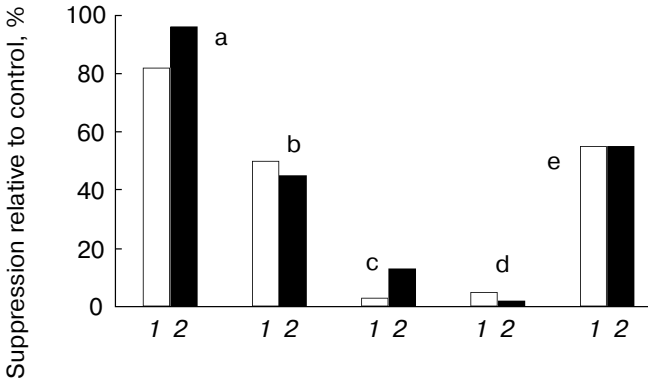

Fig. 3. Effects of indomethacin (a), esculetin (b), 5-HETE (c), 12-HETE (d), and 15-HETE (e) on the incorporation of [^{3}H]thymidine (*1*) and on the biosynthesis of PGE_2 from [1-^{14}C]AA incorporated (*2*) by A549 cells on the third day of growth.

Because the [^{3}H]thymidine incorporation into DNA of A549 cells and the biosynthesis of eicosanoids in these cells depended not only on the culture time but also on the growth density of the tumor cells, we also studied the growth inhibition with indomethacin and esculetin under conditions of 50% and of complete confluence of the culture on the fourth day of growth. In the cultures with the lower density of cell growth the [^{3}H]thymidine incorporation into DNA was suppressed with indomethacin by 60-80%, whereas on complete monolayer formation decreased this suppression to 16-20%. Note, that in the non-confluent cultures the growth-inhibiting effect of esculetin, compared with indomethacin, was less pronounced (by 42-50%), whereas in the case of the monolayer formation esculetin suppressed the incorporation of the label by 30%, i.e., its effect was more pronounced than that of the cyclooxygenase inhibitor.

Effect of bFGF on the proliferation of A549 cells and on eicosanoid biosynthesis from [^{14}C]AA incorporated. The growth modulation with bFGF was studied in the tumor cell cultures with different degree of confluence in the presence of 5% FCS and also in cultures with a shortage of serum (0.5%) in the medium. In all experiments, bFGF had no effect on the [^{3}H]thymidine incorporation into the tumor cells. However, its effect on the number of adhesive cells in the culture depended on the growth conditions. Thus, the intact cultures in the presence of 5% FCS contained (107.2 ± 10.3)·10^3 adhesive tumor cells per cm^2, whereas the cultures supplemented with bFGF contained (119.4 ± 4.7)·10^3 such cells per cm^2 (+11% compared to the control). In the medium with 0.5% FCS the stimulating effect of this growth factor was significantly higher. At similar values of the [^{3}H]thymidine incorporation in the control and experimental cultures (14.39 ± 0.79 and 14.77 ± 0.46 cpm per 10^3 cells, respectively) the number of adhesive cells in the control was (104.6 ± 5.6)·10^3 per cm^2, whereas under the influence of bFGF it was (130.0 ± 9.8)·10^3 cells per cm^2 (+24%, $p < 0.05$). A pronounced increase in the number of tumor cells in the serum-deficient medium in the presence of bFGF was also found in the cultures with the lower growth density. Note, that the stimulating effect of the growth factor in all cases was completely abolished by cyclooxygenase inhibitor indomethacin.

Under standard culture conditions bFGF did not stimulated the biosynthesis of individual eicosanoids, and this correlated with the lack of the growth factor effect on the [^{3}H]thymidine incorporation in the cultures with the different degree of the confluence. Thus, on the third day of the culture growth during the most intense synthesis of eicosanoids the growth factor effect on the [^{14}C]AA con-

version into $PGF_{2\alpha}$, 5-HETE, 12- and 15-HETE, and also into PGE_2 was −13, −1, −10, and +3%, respectively, compared to control (Fig. 2). The absence of the bFGF effect on the PGE_2 production and a poor suppression of the biosynthesis of other metabolites of [^{14}C]AA also occurred on the fourth day of the culture growth. At that time the growth factor caused a 50% suppression of the 12- and 15-HETE production in cultures without medium replacement before the introduction of the labeled precursor.

Thus, during A549 cell culture growth [^{3}H]thymidine incorporation into DNA and biosynthesis of eicosanoids from [^{14}C]AA incorporated were significantly decreasing. In the tumor cell cultures with confluence unaccomplished the inhibitors of AA metabolism, indomethacin and esculetin, and a lipoxygenase metabolite of AA, 15-HETE significantly suppressed both the [^{3}H]thymidine incorporation and the production of PGE_2. Other lipoxygenase metabolites of AA (5-HETE and 12-HETE), which displayed features of a growth factor for some tumor cells, had no effect on the A549 cell proliferation. The pronounced stimulating effect of bFGF on A549 cell proliferation in the media with low (0.5%) content of FCS was abolished by the cyclooxygenase inhibitor indomethacin.

DISCUSSION

In the oncological aspect, PGE_2 is the best studied eicosanoid. Its intense biosynthesis is found in many tumor cells of humans [12, 19] and animals [20, 21]. In tumor cells, PGE_2 is synthesized from AA with involvement of both cyclooxygenase isoforms, constitutive (COX-1) and inducible (COX-2), and the activity of the latter significantly increased during inflammation or tumor development [12]. COX-2 is also highly expressed in A549 cells [22]. Many authors have shown an important role of PGE_2 in proliferation [12, 13] and metastasizing [23] of tumor cells, apoptosis [24], immunosuppression [25], angiogenesis [26], and also its ability to stimulate the production of growth factors in the cells [26]. However, lipoxygenase metabolites of AA, various mono-HETE, have been recently found to have similar features, and their role in carcinogenesis is now studied intensively. Thus, 5-HETE actively produced by some tumor cells displays a pronounced mitogenic and antiapoptotic effect [15]. The effect of 12-HETE is directed otherwise, it markedly affects angiogenesis [17], retraction, proliferation, and migration of endothelial cells [17, 27], invasion, adhesion, and migration of tumor cells [28-30]. However, 12-HETE is also shown to be a growth-limiting agent for some tumor cells [16].

Although the profile of eicosanoids synthesized from [^{14}C]AA incorporated in normal fibroblasts of rat bone marrow stroma [18] is unlike that found in A549 human lung adenocarcinoma cells, such parameters as conversion of the labeled precursor into PGE_2, the growth-inhibiting effects of indomethacin, esculetin, and 15-HETE, and also the mitogenic effect of bFGF were similar in both systems. However, the modulating effect of indomethacin, an equipotent inhibitor of COX-1 and COX-2 [22], on proliferation of the tumor cells had some specific features. Thus, the significant suppression of the [^{3}H]thymidine incorporation with indomethacin was found by us during the early stage of growth of cultures or at the low density of adhesive cells and was absent in the case of the monolayer formation completed. This dependence of the indomethacin effect on the culture growth density seems to explain in some cases the absence of its influence on the cell proliferation stimulated by bFGF [31].

The significant decrease in the PGE_2 biosynthesis in the growing culture of A549 cells can be a result of close intercellular interactions during the monolayer formation and/or of an accumulation in the medium of its endogenous inhibitors along with an increase in the number of tumor cells. In particular, 15-HETE can be such an endogenous inhibitor which similarly suppressed in A549 cells both production of PGE_2 and [^{3}H]thymidine incorporation into DNA. Note, that 12- and 15-HETE were not produced in A549 cells on addition of [^{14}C]AA to the fresh medium, and their production was significantly inhibited with bFGF when the labeled precursor was added into the medium unchanged. These specific features of the metabolism of [^{14}C]AA introduced under different culture conditions and the growth-modulating influence of the factors which were present in the medium and secreted by the tumor cells seem to explain the significant decrease in PGE_2 biosynthesis during the late stages of the culture growth and the more intense growth of the cells after the medium had been changed. It is important that the biosynthesis of 12- and 15-HETE in neoplastic tissue in patients with different forms of lung cancer is significantly suppressed along with a pronounced spreading of the tumor to lymph nodes and other organs [32, 33].

Considering the great branching of AA cascade metabolites and their complicated interactions to one another and with growth factors, which can induce in normal and tumor cells the synthesis of PGE_2 or 12-HETE [17, 34-36], it is also necessary to study the metabolism of AA. The necessity of studies in detail of the eicosanoid metabolism may be based on the finding of the cell growth suppression with esculetin that indirectly suggests an involvement of the lipoxygenase pathway of AA metabolism in cell proliferation [37]. But our studies on the A549 cell line and the *ex vivo* testing on a cell-free homogenate of mouse lung have shown that esculetin is not a selective inhibitor of the lipoxygenase pathway; it also inhibits the production of some cyclooxygenase metabolites of AA including PGE_2, and its growth-inhibiting effect is associated with inhibition of the

biosynthesis of just this prostanoid. At present, the key role of PGE_2 in the growth control of tumor cells with active synthesis of cyclooxygenase and lipoxygenase metabolites of AA is evident, as well as its significance in the mitogenic effect of bFGF. In this connection the features of 15-HETE as of a down-regulator of the PGE_2-dependent proliferation seem to be also very important. However, the degree of involvement of other lipoxygenase metabolites of AA in the proliferation should be refined, and cell lines with the predominant synthesis of various mono-HETE seem to be the best models for such studies.

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