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Influence of plant polyphenols on metabolism of polyamines and expression of proteins, products of some oncogenes in experimental tumors

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Aim: to investigate effect of green tea polyphenolics (GTPPh) on growth of Walker W-256 rat carcinosarcoma and certain molecular pathways as possible targets of GTPPh's antitumor effect; special focus was on GTPPh's influence on polyamines metabolism, activation of the NF-κB transcription factor and expression of protein products of the NF-κB-dependent genes such as c-myc and bcl-xl.

Methods: expression of NF-κB proteins and proteins of c-myc and bcl-xl genes was measured by the methods of Western blotting and surface plasmon resonance (SPR), content of polyamines (PA) — by high-performance liquid chromatography and thin-layer chromatography, activity of polyamine oxidase (PAO) — by spectrofluorometric method, expression of ornithinedecarboxylase (ODC) — by the methods of immunohistochemistry and Western blotting. The Western blotting data were subjected to computer densitometry using the TotalLab program. Total protein content was measured by M.Bradford's methods. Statistical treatment of the numeric data was performed using Student's t-criterion. Results: We proved that GTPPh administered to the tumor-grafted animals lead to essential retardation of Walker carcinosarcoma growth. Growth retardation was accompanied by significant decrease of ODC protein expression, PAO activity and PA content in the tumor cells. On the background of Walker carcinosarcoma growth retardation, expression of p50 and p65 proteins, the NF-κB factor subunits, in the nuclei of tumor cells and expression of c-myc and bcl-xl genes producing proteins in the tumor cells were shown to be essentially diminished. Conclusions: Our data prove GTPPh to be natural inhibitors of PA biosynthesis and interconversion. Also we showed that GTPPh diminish activation of the NF-κB transcription factor and expression of protein products of the NF-κB-dependent oncogenes (c-myc and bcl-xl). The inhibition of abovementioned cascade, probably, contributes to growth retardation of the examined tumors.

Key words: mammary tumors, green tea polyphenolics, polyamines, NF-κB transcription factor, c-myc and bcl-xl.

INTRODUCTION

Last years, plant polyphenolics (PPh) were of rapt attention in connection with their discovered antitumor and anticancerogenic properties. These properties can be attributed to PPh of a lot of plant species and plant foods, for example, green tea (the most extensively studied — epigallocatechine-3-gallat), grape resveratrol, soybean genistein and daidzein and others [1-8]. In a lot of *in vitro* studies, plant PPh were found to suppress proliferation of human and animals' malignant cells, especially cells of lung, intestinal, breast, prostate tumors, hepatoma, leucosis and others [5-8]. Antitumor and anti-metastasis activities of different plant PPh were also shown in the *in vivo* experiments [6, 9, 10] and confirmed in extended epidemiological studies that demonstrated reduced levels of breast and prostate cancer morbidity in the countries where green tea and soybean foods consumption is widespread and regular [2] and in some clinical trials [11, 12].

Although antitumor molecular pathways of plant PPh are not sufficiently understood for to-

day, there is strong evidence regarding apoptosis activation in tumor cells and angiogenesis inhibition in PPh-affected tumors [10, 13, 14]. There are also some works demonstrating PPh-caused inhibition of polyamines synthesis in tumor cells [15-17].

Polyamines (spermine, spermidine, putrescine) are known as obligate agents for proliferation and growth of any cells including malignant ones. It is necessary to point out that polyamines (PA) per se and enzymes of their metabolism are considered now as targets for antitumor therapy [18-24]. Inhibition of PA synthesis in tumor cells was shown to retard proliferation of the latter and the tumor growth. A lot of foreign as well as our own works show drastic growth retardation of different kinds of experimental tumors [18-21]. This effect was recently demonstrated also in clinical trials. Especially promising should be considered the data on α-DFMO application for prevention and therapy of intestinal cancer and tumors of other localizations [22, 23].

Our previous studies show that PA impact on functioning of the NF-κB nuclear transcription factor may be one of the important pathways of tumor growth control [24-27]. We have demonstrated that PA, especially spermine, have specific affinity to p50-subunit of NF-κB and promote factor binding to specific regulatory sites of DNA (NRE-sequences) of NF-κB-dependent genes. Under intracellular PA depletion (after treatment with α-difluoromethylornithine, α-DFMO — a specific inhibitor of the key enzyme of PA biosynthesis — ornithine-decarboxylase, ODC) we observed growth retardation in MCF-7 cell culture line of human breast cancer and diminished activity of NF-κB classic form (p50/p65 heterodimer) in these cells. Introduction of putrescine into culture medium restituted PA level and NF-κB activity in these cells as well as MCF-7 cell growth rate [24, 26]. These data indicate that one of the possible molecular pathways of PA influence on cell proliferation is PA involvement in control of NF-κB activity and, through this, in transcription control of NF-κB-dependent oncogenes, for example, *c-myc, bcl-xl, cox-2, inos* etc. At the same time, molecular pathways of PA involvement in growth processes are not clear.

The drawback of α-DFMO and some other synthetic inhibitors of PA metabolism is their certain side effects such as ototoxic action, activation of cyclooxygenase (COX-2) and inducible NO-syntase (iNOS). Because of this, search for natural compounds able to inhibit PA synthesis in tumor cells but devoid of side effects is important in prospect of their further application in clinical oncology.

The aim of the present work was: to study the green tea PPh effect on growth of Walker (W-256) rat mammary carcinosarcoma and on certain molecular pathways; of special interest were PPh influence on PA metabolism, NF-κB activation and expression of protein products of certain NF-κB-dependent genes in W-256 cells.

MATERIALS AND METHODS

Experimental model. The grafted strain of Walker (W-256) rat mammary carcinosarcoma maintained in R.E.Kavetsky Institute of experimental pathology, oncology and radiobiology of NAS of Ukraine (IEPOR) by *in vivo* passages was used. The experiments were performed on sexually mature female rats bred in IEPOR vivarium. Tumors were grafted to the animals subcutaneously with cell suspension (2 - 4,5×10^6 cells in 0.5 ml of isotonic saline per 1 animal).

The extract of green tea PPh (GTPPh) obtained from S.Durmishidze Institute of biochemistry and biotechnology of NAN of Georgia was used in the experiments. In the experimental groups, the animals consumed GTPPh (1 mg/ml solution in drinking water) instead of drinking water from the day of tumor transplantation up to the day of sacrificing. Consumed volume of GTPPh solution in the experimental groups was practically equal to volume of drinking water consumed by the control animals and amounted in average 25±3 ml/day per one animal. 11–12 days after tumor transplantation, the animals were scarified under deep ether narcosis, tumors were extirpated and weighted. All *in vivo* experiments were carried out in accordance to the "European convention for the protection of vertebrate animals used for experimental and other scientific purposes", 1986.

Biochemical and molecular-biological investigations. To evaluate expression of proteins, cellular and nuclear extracts were prepared from tumors tissue using the method [28]. Expression of NF-κB proteins and proteins of NF-κB-dependent genes *c-myc* and *bcl-xl* was evaluated by the methods of Western-blotting [29, 30] and surface plasmon resonance (SPR) [31]. The SPR experiments were carried out using Biosupplar-5 and Plasmon SPR-05 SPR-spectrometers produced in Institute of semiconductors physics of NAS of Ukraine [32]. Western blotting analysis, SPR and immunohistochemical studies were performed using corresponding monoclonal antibodies produced by Santa Cruz Inc. (USA). To measure polyamines

TABLE 1

Effect of GTPPh on polyamines concentration in cellular extracts of Walker carcinosarcoma; M±m; nM/mg of total protein

Polyamines	Control	GTPPh
Putrescine	6,8 ± 1,1	2,9 ± 0,9*
Spermidine	19,5 ± 1,2	14,2 ± 0,8*
Spermine	14,8 ± 0,8	11,9 ± 0,6*

*Remark: * — P<0,05.*

TABLE 2

Effect of GTPPh on polyamines concentration in nuclear extracts of Walker carcinosarcoma; M±m; nM/mg of total protein

Polyamines	Control	GTPPh
Putrescine	0,9 ± 0, 07	0,5 ± 0,04*
Spermidine	6,8 ± 0,20	4,4 ± 0,2*
Spermine	8,2 ± 0,40	5,6 ± 0,2*

*Remark: * — P<0,05.*

content in cytoplasm and nuclear extracts of tumor cells, high-performance liquid chromatography [33] and thin-layer chromatography were used with farther spectrofluorimetry [34]. Hydrochlorides of polyamines produced by Calbiochem (USA) were used as standards polyamines. Polyamine-oxidase (PAO) activity in tumor tissue was evaluated by spectrofluorimetric method [35], ODC expression — by Western blotting [30] and immunohistochemical method [36]. The Western blotting data were subjected to computer densitometry using the TotalLab program. Total protein content was measured by M.Bradford's method [37]. Statistical treatment was performed using Student's t-criterion.

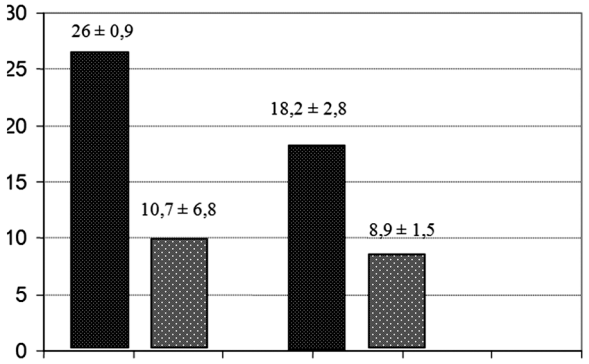

Fig. 1. Effect of GTPPh on growth rate (average mass) of Walker carcinosarcoma.

RESULTS AND DISCUSSION

Our experiments demonstrated significant growth retardation of Walker carcinosarcoma in the animals receiving GTPPh in the mode described above (fig.1). This effect was accompanied by essentially diminished ODC protein expression, together with reduced PAO activity (enzyme, involved in polyamines interconversion) and polyamines content (tables 1, 2) in the cellular and nuclear extracts of Walker carcinosarcoma. Diminished ODC expression was shown not only by Western blotting but also by immunohistochemical method.

Since GTPPh consumption led to decrease of both ODC expression and PAO activity, our experiments provide a ground to suggest that GTPPh are natural inhibitors of both biosynthesis and

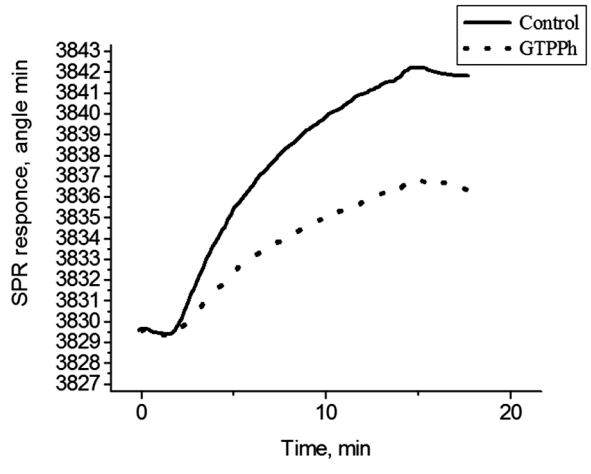

Fig. 2. Effect of GTPPh on c-myc protein expression in Walker carcinosarcoma cells (typical SPR sensograms).

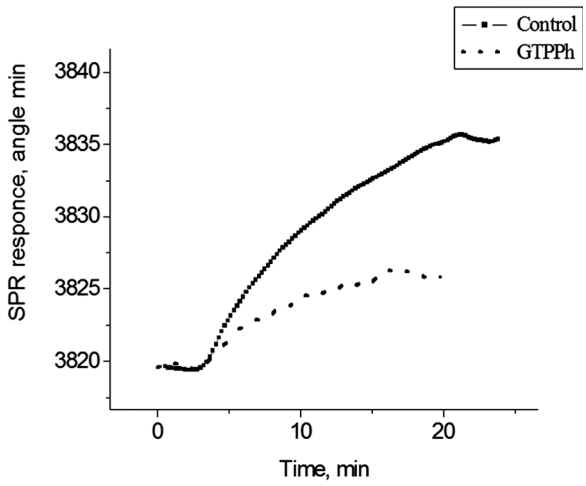

Fig. 3. Effect of GTPPh on Bcl-XL protein expression in Walker carcinosarcoma cells (typical SPR sensograms).

inerconversion of PA. GTPPh ability to inhibit PAO activity is crucial because this inhibition leads to disruption of the transformation of acetyl-PA to PA, which are able to restitute intracellular PA pool and through this to abolish growth retardation caused by inhibitors of PA biosynthesis alone.

Also we demonstrated that growth retardation of Walker carcinosarcoma is accompanied by strong increase of nuclear p50 and p65 proteins– subunits of classic heterodimer form of NF-κB transcription factor. This fact we consider of the utmost importance. Indeed, in our experiments with classic ODC inhibitor — α-DFMO — on various tumor models we observed increase of p50 content in the nuclei of tumor cells [24, 26] and decrease in NF-κB activity. Diminished functional activity of NF-κB under these conditions can be explained by discrepancy in the levels of p50 and p65 and as a result — preferential formation of p50/p50 homodimer, which is a non-active form of NF-κB under low PA concentration. So, GTPPh-caused reduction in nuclear content of both p50 and p65 shows qualitative difference in effects of GTPPh and α-DFMO on NF-κB.

Some authors state that p50/p50 homodimer under PA deficiency is not able to bind DNA and consequently does not enhance transcription [38, 39]. Nevertheless, even if p50/p50 — homodimer binds to DNA, the p50/p50 competes in binding with the classic p50/p65 heterodimer and impedes effective transcription.

We observed essential decrease of expression of protein products of some NF-κB-dependent oncogenes, such as *c-myc* (for 40%) и *bcl-xl* (for 67%) in Walker carcinosarcoma under GTPPh (fig. 2, 3).

So, it seems plausible that antitumor effect of GTPPh is mediated by PA- and NF-κB-dependent signal pathways.

CONCLUSION

Green tea polyphenolics, in dose used, are the natural inhibitors of polyamines biosynthesis and interconversion. We also demonstrated that polyphenolics diminish the NF-κB transcription factor activation and expression of protein products of NF-κB-dependent oncogenes — *c-myc* and *bcl-xl*. All of the abovementioned effects contribute to growth retardation of Walker W-256 carcinosarcoma observed in our experiments.

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С.П.Залєток, О.А.Орловський, С.В.Гоголь, О.А.Самойленко, О.О.Кленов, І.В.Малицька, Л.Гулуа. Вплив рослинних поліфенолів на метаболізм поліамінів та експресію білків, продуктів деяких онкогенів в експериментальних пухлинах. Київ, Україна, Тбілісі, Грузія.

Ключові слова: *пухлини молочної залози, поліфеноли зеленого чаю, поліаміни, фактор транскрипції NF-κB, c-myc и bcl-xl.*

Мета: дослідити вплив поліфенолів зеленого чаю (ПФЗЧ) на ріст карциносаркоми Уокер (W-256) у щурів, а також вивчити молекулярні механізми їх протипухлинної дії, зокрема вплив на метаболізм поліамінів, активацію фактора транскрипції NF-κB та експресію білків, продуктів NF-κB-залежних генів c-myc та bcl-xl. Методи дослідження: експресію білків фактора NF-κB і білків генів c-myc та bcl-xl визначали методами Western-blotting та поверхневого плазмонного резонансу (ППР). Вміст поліамінів (ПА) визначали методами високоефективної рідинної хроматографії і тонкошарової хроматографії. Активність

поліаміноксидази (ПАО) визначали спектрофлуориметричним методом, експресію орнітиндекарбоксилази (ОДК) — імуногістохімічним методом та методом Western-blotting. Дані Western-blotting піддавали математичній обробці за допомогою програми TotalLab. Визначення білка проводили методом M.Bradford. Статистичну обробку даних проводили за t-критерієм Стьюдента. Результати: Виявлено, що споживання тваринами ПФЗЧ призводить до істотного пригнічення росту карциносаркоми Уокер. Гальмування росту пухлин супроводжувалося значним зменшенням експресії білка ОДК, активності ПАО та концентрації ПА в пухлинних клітинах. На фоні гальмування росту карциносаркоми Уокер в ядрах пухлинних клітин істотно знижується експресія білків p50 і p65 — субодиниць фактору NF-κB, а також зменшується рівень експресії білків, продуктів генів c-myc та bcl-xl. Висновки: Одержані дані свідчать, що ПФЗЧ є природними інгібіторами біосинтезу та інтерконверсії ПА, а також зменшують активацію фактора транскрипції NF-κB та експресію білкових продуктів NF-κB-залежних онкогенів (c-myc и bcl-xl), що, імовірно, надає свій внесок у виявлене нами гальмування росту досліджуваних пухлин.

С.П.Залеток, А.А.Орловский, С.В.Гоголь, Е.А.Самойленко, О.А.Кленов, И.В.Малицкая, Л.Гулуа. Влияние растительных полифенолов на метаболизм полиаминов и экспрессию белков, продуктов некоторых онкогенов в экспериментальных опухолях. Киев, Украина, Тбилиси, Грузия.

Ключевые слова: *опухоли молочной железы, полифенолы зеленого чая, полиамины, фактор транскрипции NF-κB, c-myc и bcl-xl.*

Цель: исследовать влияние полифенолов зеленого чая (ПФЗЧ) на рост карциносаркомы Уокер (W-256) у крыс, а также изучить молекулярные механизмы их противоопухолевого действия, в частности влияние на метаболизм полиаминов, активацию фактора транскрипции NF-κB и экспрессию белков, продуктов NF-κB-зависимых генов c-myc и bcl-xl. Методы исследования: экспрессию белков фактора NF-κB и белков генов c-myc и bcl-xl определяли методами Western-blotting и поверхностного плазмонного резонанса (ППР). Содержание полиаминов (ПА) определяли методами высокоэффективной жидкостной хроматографии и тонкослойной хроматографии. Активность полиаминоксидазы (ПАО) определяли спектрофлуориметрическим методом, экспрессию орнитиндекарбоксилазы (ОДК) — иммуногистохимическим методом и методом Western-blotting. Данные Western-blotting были подвергнуты математической обработке при помощи программы TotalLab. Определение белка проводили методом M.Bradford. Статистическую обработку данных проводили по t-критерию Стьюдента. Результаты: Установлено, что потребление животными ПФЗЧ приводит к существенному угнетению роста карциносаркомы Уокер. Торможение роста опухолей сопровождалось значительным снижением экспрессии белка ОДК, активности ПАО и уменьшением концентрации ПА в опухолевых клетках. Выявлено, что на фоне торможения роста карциносаркомы Уокер в ядрах опухолевых клеток существенно снижается экспрессия белков p50 и p65 — субъединиц фактора NF-κB, а также уменьшается уровень экспрессии белков, продуктов генов c-myc и bcl-xl. Выводы: Полученные данные свидетельствуют, что ПФЗЧ являются природными ингибиторами биосинтеза и интерконверсии ПА, а также снижают активацию фактора транскрипции NF-κB и уменьшают экспрессию белковых продуктов NF-κB-зависимых онкогенов (c-myc и bcl-xl), что, вероятно, привносит свой вклад в обнаруженное нами торможение роста исследуемых опухолей.

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