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Crosstalk Between Normal and Tumoral Brain Cells

Effect on Sex Steroid Metabolism

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The present article shows for the first time that two cell lines derived respectively from a rat glioma (C6 cell line) and from a human astrocytoma (1321N1 cell line) are able to convert testosterone and progesterone into their corresponding 5α-reduced metabolites dihydrotestosterone and dihydroprogesterone. Moreover, both cell lines are also able to convert these metabolites further into their corresponding 3α-OH derivatives, 5α-androstan-3α, 7β-diol (3α-diol) and tetrahydroprogesterone. On the basis of these observations, the possibility that secretory products of normal and tumoral brain cells might be able to influence steroid metabolism occurring in the two glial cell lines previously mentioned as well as in fetal rat neurons and in neonatal rat type 1 astrocytes has been considered. To this purpose, cultures of the different cellular types have been exposed to the conditioned medium in which the other cells were grown. The results obtained indicate that:

1. Neurons are able to stimulate, in a statistically significant fashion, the formation of dihydrotestosterone (DHT), 3α-diol, and tetrahydraprogesterone (THP) in C6 cells.
2. Type 1 astrocytes, on the contrary, are unable to modify steroid metabolism in C6 cells.
3. C6 cell product(s) decrease(s) the formation of DHP in type 1 astrocytes, without modifying that of DHT.
4. C6 cells do not influence the metabolism of testosterone (T) and progesterone (P) in neurons.

In conclusion, the present observations show that the conditioned medium of normal neurons is able to increase the metabolism of testosterone and progesterone occurring in a tumoral glial cell line, and that the conditioned media of the two tumoral cell lines analyzed are able to decrease the conversion of P into DHP occurring in normal type 1 astrocytes. The surprising result that these conditioned media do not alter the formation of DHT is discussed. Work is presently in progress to identify the principle(s) responsible respectively for the activating and inhibiting actions here described.

Key Words: Rat glioma; human astrocytoma; neurons; astrocytes; testosterone; progesterone.

Introduction

Previous work of this laboratory has shown that both neurons and glial cells (type 1 and type 2 astrocytes, oligodendrocytes) in culture are able to convert androgens and progestagens into their corresponding 5α-reduced metabolites, since both types of cells possess the enzymes 5α-reductase (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD) *(1,2)*. Through the action of these enzymes, testosterone (T) is converted into 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT), and subsequently into 5α-androstan-3α,17β-diol (3α-diol). Similarly, progesterone (P) is converted into 5α-pregnan-3,20-dione (dihydroprogesterone, DHP), and then into 5α-pregnan-3α-ol-20-one (tetrahydroprogesterone, THP).

In a previous paper, it was also shown that the principle(s) released in the culture medium by neurons may stimulate both the 5α-R and the 3α-HSD present in type 1 astrocytes. On the contrary, there was no effect of astrocytic secretions on the 5α-R–3α-HSD complex present in neurons *(3)*. On the basis of these observations, it was deemed of interest to investigate whether factors produced by normal fetal rat neurons might be able to modify the metabolism of T and P in tumors derived from glial cells. To this purpose, two glial cell lines (the C6 rat glioma and the 1321N1 human astrocytoma) have been used. First of all, it was necessary to establish whether these two tumoral cell lines are able to metabolize sex steroids via the 5α-R–

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3α-HSD pathway. Subsequently, the effects of the conditioned media (CM) obtained from cultures of fetal rat neurons on the metabolism of T and P in the C6 rat glioma cell line were analyzed. In parallel experiments, the effects possibly exerted by the CM of type 1 astrocytes on steroid metabolism occurring in the two tumoral cell lines (C6 and 1321N1) were also evaluated. Finally, it was decided to verify whether factors secreted by the tumoral cells into their incubation media might influence steroid metabolism occurring, respectively, in neurons and in type 1 astrocytes.

Results

Table 1 shows that the rat glioma C6 and the human astrocytoma 1321N1 cell lines are able to convert T and P into their corresponding 5α-reduced metabolites. In the table, the amounts of the metabolites formed in the two tumoral cell lines are compared to those found in cultures of type 1 astrocytes and of neurons obtained from the brains of neonatal and fetal rats, respectively.

In C6 cells, the formation of DHT from T is significantly higher than that occurring in type 1 astrocytes, but significantly lower than that observed in neurons. In these tumoral cells, the formation of 3α-diol from DHT is 10 times lower than that found in type 1 astrocytes, and similar to that found in neurons. The conversion of P into DHP follows a pattern similar to that described for the conversion of T into DHT, but with higher yields. Similarly to what has been observed when DHT was used as substrate, the conversion of DHP into THP is 10 times lower than that observed in type 1 astrocytes.

The profile of steroid metabolism appears different in the case of the 1321N1 cells. In this cell line, like in C6 cells, the formation of DHT is significantly lower than that occurring in neurons; in this case, however, the formation of DHT is similar to that found in astrocytes and the formation of 3α-diol is significantly lower than that found in astrocytes. In this cell line, the formation of DHP from P is significantly higher than that recorded in type 1 astrocytes, and much lower (about one-third) than that occurring in neurons. In this cell line, the formation of THP is almost 20 times lower than that seen in type 1 astrocytes, and slightly higher than that observed in neurons.

A comparison between the metabolism of T and P occurring in C6 and in 1321N1 cells shows that the formation of DHT is significantly higher in C6 than in 1321N1 cells, whereas the formation of DHP is almost identical in the two cell lines. With regard to the activity of the 3α-HSD, it appears that the formation of both 3α-diol and THP is not significantly different in C6 and in 1321N1 cells.

Figure 1(A) shows that the exposure of C6 cells to the CM of normal fetal rat neurons results in a significant stimulation of the formation of DHT, but not of DHP from their respective substrates. The exposure of C6 cells to the CM of neurons also brings about a significant increase of the conversion of DHT into 3α-diol and of DHP into THP (Fig. 1B). In the reverse experiment, in which neurons were exposed to the CM of C6 cells, the formation of DHT, DHP, 3α-diol, and THP from the respective substrates remained unmodified (Fig. 1A,B).

Figure 2 (A,B) shows that in C6 cells exposed to the CM of type 1 astrocytes, there are no modifications of the formations of DHT, DHP, 3α-diol, and THP from the respective substrates. In the reverse experiment, in which the effects possibly exerted by factors secreted by C6 cells were studied on the metabolism of T and P occurring in type 1 astrocytes, it was observed that the CM of C6 cells was unable to modify the formation of DHT; on the contrary, a statistically significant inhibition of the formation of DHP is observed in type 1 astrocytes exposed to the CM of C6 cells (Fig. 2A). There is no effect of the tumoral CM on 3α-diol and THP formation from DHT and THP, respectively (Fig. 2B).

As in C6 cells, also in the 1321N1 cells, the addition of the CM of type 1 astrocytes does not modify the formation of any of the 5α-reduced metabolites measured (DHT, DHP, 3α-diol, and THP; Fig. 3A,B). However, in line with the findings observed when exposing type 1 astrocytes to the CM of the C6 cells (Fig. 2A), the exposure to the CM of the 1321N1 astrocytoma cell line also induces a statistically significant decrease in the formation of DHP in type 1 astrocytes (Fig. 3A), without any modification in the formation of DHT (Fig. 3A), 3α-diol, and THP (Fig. 3B).

Discussion

The present data show for the first time that two cell lines derived respectively from a rat glioma (C6 cell line) and from a human astrocytoma (1321N1 cell line) are able to convert T and P into their corresponding 5α-reduced metabolites DHT and DHP. Moreover, both cell lines are also able to convert these metabolites further into their corresponding 3α-OH derivatives, 3α-diol, and THP. It is important to underline also that in these tumoral cell lines, like in other nervous structures and tissues possessing 5α-R activity, P seems to be the preferential substrate for the enzyme *(1,2,4)*. It is also interesting to note that the present study has shown that there are quantitative differences between the metabolic abilities of the two tumoral cell lines. These differences may be ascribed to several factors. First of all the two cell lines derive from two different species (rat vs human); moreover, their cellular composition is different (a mixed glia tumor vs astrocytoma). For reasons that will be discussed below, it appears particularly significant to note that the magnitude of conversion of P over T is much higher in the 1321N1 cell line (about 20 times) than in C6 cells (about 3 times). This ratio in C6 cells is similar to the one found in normal astrocytes and neurons (about 3–4 times).

The present data also indicate that when added to the C6 cells, the CM of fetal rat neurons is able to stimulate, in a statistically significant fashion, the formation of DHT, but

Table 1
Formation of 5α-Reduced Metabolites of T and P in Tumoral and Normal Cerebral Cells[a]

	DHT mean ± SEM *n*	3α-diol mean ± SEM *n*	DHP mean ± SEM *n*	THP mean ± SEM *n*
C6	14.7 ± 1.2[b,c] 10	87.3 ± 6.2[b] 4	48.4 ± 3.8[b,c] 9	103.0 ± 5.2[b] 4
1321N1	2.0 ± 0.3[c,d] 4	45.9 ± 0.9[b] 4	42.8 ± 3.6[b,c] 5	56.9 ± 1.7[b] 4
AI	4.9 ± 0.4 8	950.1 ± 47.6 4	18.8 ± 1.6 5	1031.8 ± 101.1 4
Neurons	28.9 ± 1.7 8	79.9 ± 3.4 6	123.0 ± 8.1 4	39.5 ± 2.1 4

[a]Data are expressed in ng of steroid formed/mg of protein.
[b]$P < 0.01$ vs type 1 estrocytes (AI).
[c]$P < 0.01$ vs neurons.
[d]$P < 0.01$ vs C6.

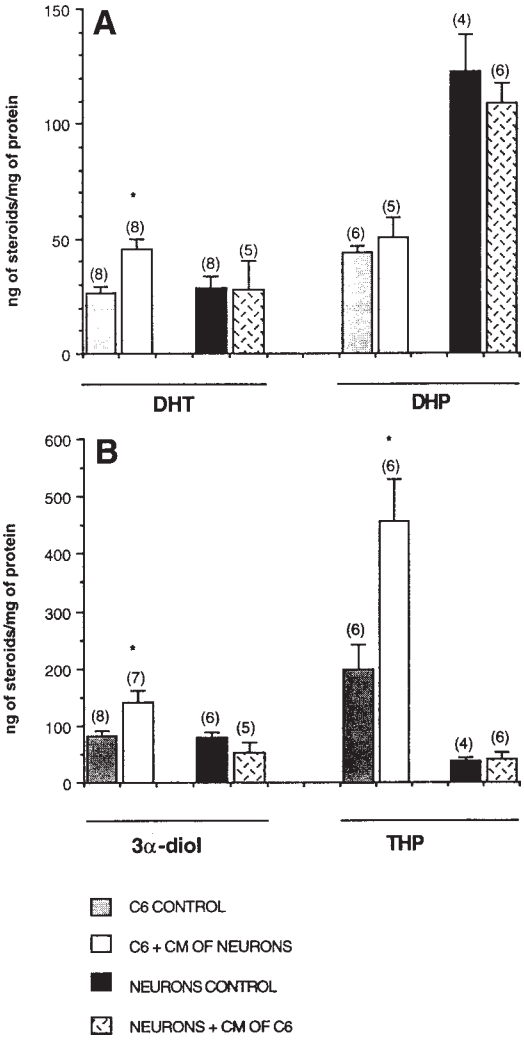

Fig. 1. Formation of DHT, DHP (**A**), 3α-diol, and THP (**B**) in neurons and in C6 cells exposed for 48-h to the conditioned media (CM) of the other cell type. CM have been obtained after 2 d of culture. Numbers in parentheses represent the numbers of determinations performed. *$P < 0.01$ vs the corresponding controls. The data are expressed as means ± S.D.

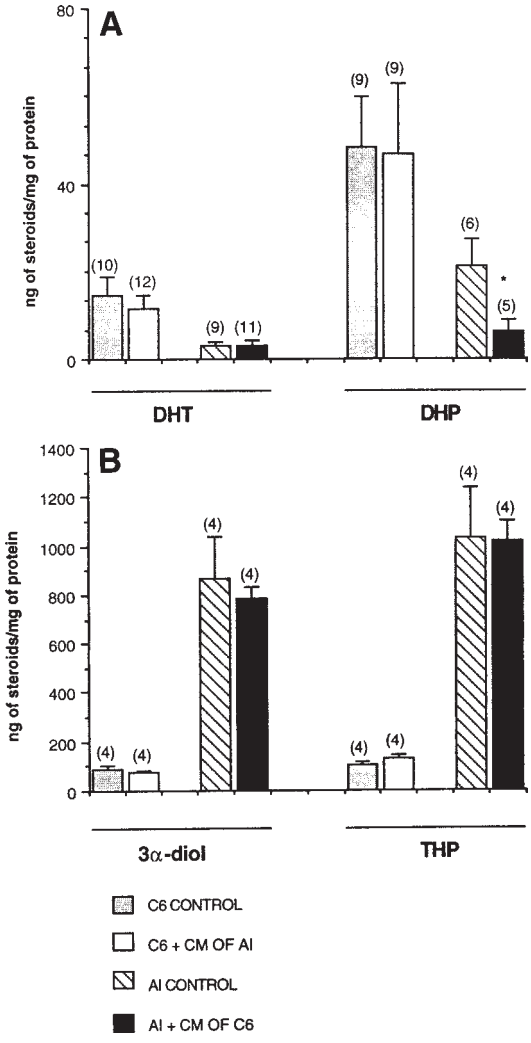

Fig. 2. Formation of DHT, DHP (**A**), 3α-diol, and THP (**B**) in type 1 astrocytes and in C6 cells exposed for 48-h to the conditioned media (CM) of the other cell type. CM have been obtained after 2 d of culture. Numbers in parentheses represent the numbers of determinations performed. *$P < 0.01$ vs the corresponding controls. The data are expressed as means ± S.D.

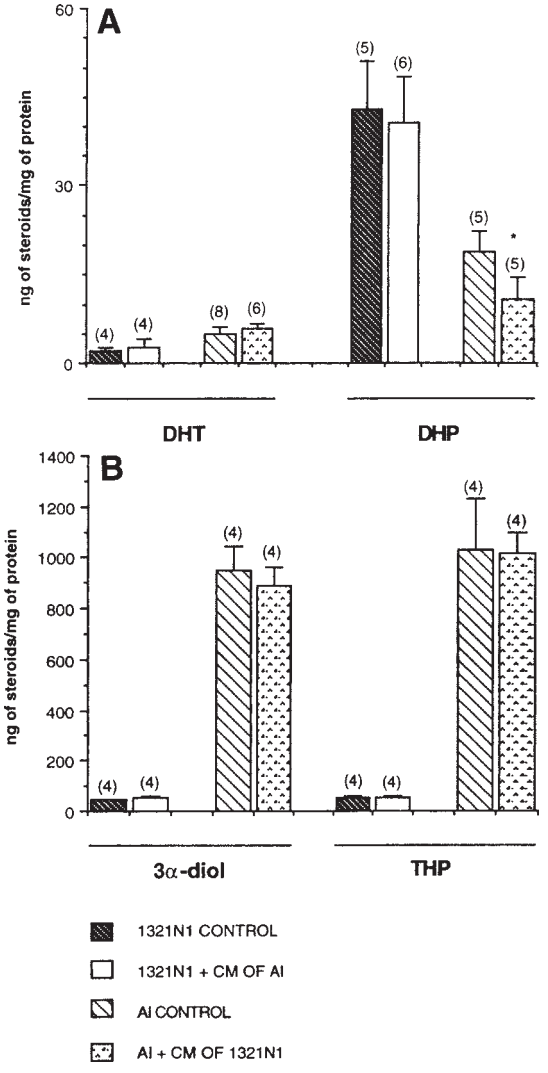

Fig. 3. Formation of DHT, DHP (**A**), 3α-diol, and THP (**B**) in type 1 astrocytes and in 1321N1 cells exposed for 48-h to the conditioned media (CM) of the other cell type. CM have been obtained after 2 d of culture. Numbers in parentheses represent the numbers of determinations performed. *$P < 0.01$ vs the corresponding controls. The data are expressed as means ± S.D.

not that of DHP, whereas the formations of both THP and 3α-diol are significantly enhanced. These findings are consistent with previous observations of this laboratory, which have indicated that the CM of fetal rat neurons induces an increase in the formation of DHT and 3α-diol in cultures of normal rat neonatal astrocytes *(3)*. The present data seem to implicate that neuronal principle(s) is/are able to influence sex steroid metabolism not only in physiological glial cells, but also in glial-derived tumoral cells.

The present study was not designed to investigate the chemical nature of the principle(s) responsible for the neuronal stimulation of steroid metabolism in tumoral glial cells. However, previous data have shown that the 5α-R activity present in normal glial cells is stimulated by the exposure to 8br-cAMP, whereas it is not sensitive to phorbol esters *(5)*. There is evidence showing also that C6 glioma cells are sensitive to cAMP, since an increase in the levels of this second messenger induces strong morphological changes *(6)*, as well as an increase in glial fibrillary acidic protein (GFAP) gene espression also in these cells *(7)*. These data suggest that the factor(s) originating in the neuronal CM might act via the cAMP pathway.

In the experiments in which the possible influences exerted by the CM of neonatal astrocytes have been evaluated on steroid metabolism occurring in the C6 and in the 1321N1 cell lines, it has been found that type 1 astrocyte CM is not able to modify the activities of either the 5α-R or the 3α-HSD in these cell lines, independently of whether T or P, and DHT or DHP have been used as the substrates. This seems to exclude the possibility that astrocytes might secrete factors able to influence T and P metabolism in CNS-derived cells.

A new finding emerging from the present data is that the CM of both C6 and 1321N1 cells are able to decrease, in a statistically significant way, the formation of DHP in type 1 astrocytes. This observation deserves several comments. First of all, it must be pointed out that this appears to be a specific effect of tumoral glial cells on the astrocytes, since a similar effect was not observed in neurons (at least for the C6 cells, which is the only cell line examined in this series of experiments). Second, it is important to underline that the inhibitory effect occurs only on P and not on T metabolism. An explanation of this surprising and possibly important finding must consider first of all the fact that both C6 and 1321N1 cells form less DHT from T than DHP from P; this might suggest that the formation of DHT cannot be further decreased. However, this hypothesis does not seem acceptable, because especially the C6 cells form DHT in amounts that might be sensitive to an inhibiting stimulus. Another hypothesis is that the enzyme present in these tumoral cells and converting P might be different from the one converting T. Since it is well known that the two isoforms of the 5α-R so far cloned are both able to convert T and P, and that for both isozymes, P appears to be the preferential substrate *(8,9,10)*, one might hypothesize the existence of a third 5α-R isoform. In support to this hypothesis, Melcangi et al. *(11)* have shown that a pluripotential CNS stem cell derived from the mice striatum, when induced to differentiate into glial elements, acquires the ability to transform P into DHP 4 d earlier than that of converting T into DHT. Obviously, this phenomenon cannot be explained on the basis of enzymes possessing both T- and P-converting activities, like the two 5α-R isoforms so far cloned. The hypothesis of the possible existence of a third 5α-R isozyme emerges also from other data presented in the present work. One may quote the results shown in Table 1, which show that in 1321N1 cells the ratio DHP/DHT is much higher that in any other cell studied in the present experiments; moreover, exposure of C6 cells to the CM of neurons results in a stimulation of the formation of DHT, but not of DHP; finally, in astrocytes exposed to the CM of

the two tumoral cell lines studied, the formation of DHP was significantly decreased, whereas there was no variation in that of DHT. The hypothesis of the existence of a third 5α-R isoform in the brain finds support in previous observations performed by Lephart *(12)*, which have shown that finasteride is able to block the 5α-R activity of the prostate, without interfering with the same activity in the brain. Moreover, it has been underlined that at the pituitary level, the low 5α-R mRNA content does not reflect the high and finasteride-resistant enzymatic activity present in the gland, pointing once more to the possibility that the 5α-R activity of this structure might be attributed to an unidentified 5α-R isoform *(13)*.

The biological meaning of the effects exerted by tumoral cells on the steroid metabolism of their normal counterparts is not understood at present. However, it is important to underline that it has been recently reported by this group that DHP increases and THP inhibits GFAP gene expression in type 1 astrocytes *(14)*. It is important to remember that GFAP is an intermediate filament protein, which is involved in the control of growth and differentiation of the astrocytes *(15–17)*. It is then obvious that any variation of the action of the enzymes forming these two steroids may affect the synthesis of GFAP, and consequently alter astrocyte morphology and function. One may consequently hypothesize that the tumor, by indirectly altering the formation of GFAP, might change the responsiveness of the astrocytes to tumor invasiveness. As a corollary of the present results, one might be tempted to suggest that sex steroids might be important for the growth of astrocytomas and gliomas. Unfortunately, the data on the presence of steroid receptors in these types of tumors are very scanty and do not allow any conclusions. P receptors, however, have been shown to be present in C6 glioma cells *(18)*, like in normal astroglial elements *(19)*. Similarly to what it has been reported for meningiomas, progestagens and antiprogestagens might be considered for the treatment of glioma and astrocytomas (*20,21*).

In conclusion, the present data show that the CM of normal fetal rat neurons is able to increase the metabolism of T and P in a tumoral glial cell line. Interestingly, the CM of two tumoral cell lines derived from the glia (C6 and 1321N1) is able to influence the metabolism of P (but not of T) in normal type 1 astrocytes. On the basis of this and other observations discussed in the article, the possibility of the existence of a third 5α-R isoform is suggested. Studies are presently in progress to identify and characterize the factors involved in the bidirectional crosstalks between normal and tumoral cerebral cells.

Materials and Methods

Animals

Primary cell cultures of neurons and type 1 astrocytes were prepared from the brain of fetal or neonatal Sprague-Dawley rats (Charles River, Calco, Italy) maintained in animal quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 6.30 h).

Cell Cultures

Neurons

Primary cell cultures were prepared as previously described *(22)*. For the isolation of the neurons, the cerebral hemisferes of 14-d-old rat embryos were dissected and transferred to a sterile Petri dish containing phoshate-buffered saline (PBS). After removal of the meninges and mechanical dissociation through a needle, performed in Dulbecco's Modified Eagle's Medium (DMEM) (Biochrom, Life Technologies, Italy) supplemented with 20% heat-inactivated FCS (Gibco, Italy), the cellular suspension was plated on the poly-L-lysine-coated Petri dishes (2 mL each). This was prepared immediately before use by dissolving 10 mg of poly-L-lysine (Sigma, Italy, type VII B, PM 70000) in 1 L of distilled water and sterilized by Millipore filtration. Four milliliters of poly-L-lysine solution were added to each Petri dish and incubated overnight in a CO_2 incubator (37°C, 95% air–5% CO_2). After removal of the poly-L-lysine solution, the plates were incubated in a CO_2 incubator (37°C, 95% air - 5% CO_2) for 1 h with 2 mL/each of DMEM supplemented with 20% heat-inactivated fetal calf serum (FCS), 50 U/mL penicillin, and 50 µg/mL streptomycin. Three Petri dishes of 6-cm diameter were used for each brain. After 1 d "in vitro" (d.i.v.) in a CO_2 incubator (37°C, 95% air–5% CO_2), the DMEM was removed and replaced by "Sato" medium: 5 µg/mL bovine insulin (Sigma), 50 µg/mL human transferrin (Sigma), 20 nM P (Sigma), estradiol 0.01 n*M* (Sigma), 100 µM putrescine dihydrocloride (Sigma), and 30 n*M* sodium selenite (Merck) in DMEM. In these conditions, neurons are the predominant cell type. It is obvious that the presence of 20 n*M* of P cannot influence the transformation of T and P, respectively, into DHT and DHP, since the amounts of the labeled substrates used were many times higher.

Type 1 Astrocytes

Type 1 astrocytes were prepared from primary cultures of mixed glia, which were obtained from the cerebral cortex of 1- to 2-d-old rats, as previously described (*1*). The cells were initially plated at high density (20×10^6 cells) in 75-cm^2 flasks. The mixed glial cells were then cultured for 2 wk, and the bedlayer, which consists of type 1 astrocytes, was separated by shaking. The cultures were washed one time with DMEM supplemented with 10% heat-inactivated FCS, 50 U/mL penicillin, and 50 µg/mL streptomycin, and incubated for 2 h in a CO_2 incubator (37°C, 95% air–5% CO_2). The flasks were shaken overnight in a thermostatic room at 37°C at 250 rpm on a rotary horizontal shaker. The medium was discarded, and the cellular bedlayer was washed once with fresh medium and a second time, very quickly, with trypsin/EDTA PBS solution (0.05/0.02 % w/v)

not containing Ca^{2+} and Mg^{2+}. The trypsinized cells were then centrifuged and replated in DMEM/10% FCS in 6 cm Petri dish (about 1.5×10^6/dish).

C6 and 1321N1 Cell Lines

C6 rat glioma cells (a mixed population of cells which represent a widely used model of transformed or quasi-normal immature rat glial cells) and 1321N1 human astrocytoma cells (a glioblastoma multiform that is the most common and the most malignant tumor of astrocytic origin in human adults) were obtained from the European Collection of animal cell cultures. C6 cells were grown in Ham's F-12 (Biochrom) supplemented with 1% penicillin/streptomycin, 2 m*M* L-glutamine and 10% heat-inactivated FCS. 1321N1 cells were grown in DMEM supplemented with 1% penicillin/streptomycin, 2 m*M* L-glutamine, and 10% heat-inactivated FCS. Both types of cells were seeded in 6-cm Petri dish (about 5×10^5/dish) and used at 80% confluence.

Conditioned Medium (CM)

The 48-h CM were obtained from cultures of rat type 1 astrocytes, rat neurons, C6 glioma, or 1321N1 astrocytoma cells. In the case of experiments using C6 and neurons, chemical-defined medium was utilized; in the case of experiments using C6 and type 1 astrocytes or 1321N1 and type 1 astrocytes, DMEM supplemented with 10% of FCS was used. The media were centrifuged at 1000*g* to eliminate the cells eventually present and added to the cultures of the different cell types. Cultures growing in fresh medium were utilized as controls. After 48 h of exposure, cultures of different types of cells were used in the enzymatic assays.

Incubation Procedures and Detection of Metabolites

The incubations with [^{14}C] T have been performed as previously described *(22)*. Briefly, cells have been scraped, sonicated by Microson^{TM} ultrasound sonicator (10% of output power for 10 s), and incubated in 250 µL of Krebs-Ringer buffer solution in the presence of a NADPH generating system (NADP, disodium salt, Boehringer Mannheim, $9.32 \times 10^{-3}M$; glucose 6-phosphate, disodium salt, Boehringer Mannheim, Italy, $11.76 \times 10^{-2}M$ and glucose 6-phosphate dehydrogenase from yeast grade 1, Boehringer Mannheim, 3.5×10^{-2} IU) and of [^{14}C] T ($3.16 \times 10^{-6}M$) (SA ~ 56.9 mCi/mmol, Amersham England) as the labeled substrate. The amounts of the generating system and of [^{14}C] T selected were used for ~100 µg of protein. Protein content was measured according to the method of Bradford *(23)*. Vials without cells provided the blanks. The incubation was carried out at 37°C for 2 h in a Dubnoff metabolic shaker, under a stream of O_2/CO_2 98:2. At the end of the incubation, the reaction was stopped by deep freezing of the samples. Tritium-labeled DHT (about 5000 dpm each) was added to each sample in order to evaluate the recovery. The metabolites formed were extracted twice with diethylether, and separated on a thin-layer silica gel-chromatography plate (Merck 60 F254,DC), eluted three times with dichloromethane/diethylether (11:1). The DHT spots were identified with iodine vapors, scraped, and the radioactivity counted in a Packard 300 C liquid scintillation spectrometer. Quench corrected DPM of the two isotopes were obtained by a calibration standard curve. In the experiments in which the formation of 3α-diol has been evaluated, the cells were incubated in the same conditions previously described, but with [^{14}C] DHT as the labeled precursor ($3.4 \times 10^{-6}M$) (SA ~ 57 mCi/mmol, Amersham England) *(1)*. The metabolites formed were extracted, separated, and the radioactivity counted as previously described.

The formation of DHP and THP was analyzed in two different sets of incubations using, respectively, [^{14}C] P and [^{14}C] DHP, as labeled precursors. [^{14}C] P (specific activity: 57.2 mCi/mmol) was obtained from Du Pont de Nemours, NEN division (Germany), and [^{14}C] DHP was prepared from [^{14}C] P by a chemical method (catalytic hydrogenation) in the Chemical Department of our university by Patrizia Ferraboschi. The incubation procedure was performed as previously described in detail *(2)*. After 2 h of incubation at 37°C in a Dubnoff metabolic shaker under a stream of O_2/CO_2 98:2, tritium-labeled DHP or THP was added to evaluate the recoveries. Then the samples were extracted twice with diethylether and separated on a thin-layer silica gel-chromatography plate (Merck 60 F_{254}, DC). In the incubations in which labeled P was used as substrate the plates were eluted one time with a mixture of benzene/methanol (95:5) and one time with a mixture of cyclohexane/*n*-butyl acetate (30:60); when labeled DHP was used, the elution was performed only one time with a mixture of benzene/methanol (95:5). The radioactivity was counted in a Packard 300 C liquid scintillation spectrometer. Crystallization to constant specific activity was performed at the beginning of the experiments, and repeated whenever necessary.

The amounts of androgen (DHT and 3α-diol) and progestagen metabolites (DHP and THP) were expressed as ng formed after 2 h of incubation/mg of protein.

Statistics

Statistical evaluation was performed either by a two-way analysis of variance (ANOVA), using Tukey's test for multiple comparison or by Student's *t*-test.

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