Application of (Methyl-^{11}C)-Methionine in the Multicellular Spheroid System

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Human glioma (U-343 MGa) and human colon carcinoma (HT-29) cell lines were cultured as multicellular spheroids, and the accumulations of the L- and D- enantiomers of ^{11}C-methionine were investigated. The accumulation of radioactivity in the spheroids was expressed as relative counts, by dividing the radioactivity measured in the spheroid with the radioactivity of the same volume of the incubation medium. The experiments were verified using ^{14}C-labeled L- and D-methionine. The influence of spheroid volume, specific activity, incubation time, washing time, and the environmental temperatures were investigated. The spheroid model was used to determine the effect of the lipoxygenase inhibitors BW A4C and AA-861, the ether-phospholipid type PAF-antagonist CV-6209 and the protein synthesis inhibitor cycloheximide on methionine uptake. The results showed that ^{11}C-L-methionine can be applied in the study of drug effects on multicellular tumor cell aggregates.

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Positron emission tomography (PET) enables quantitative in vivo determinations in man of physiological processes such as regional blood flow, a range of metabolic processes, receptor binding, and enzyme characteristics (*1*, *2*). Brain tumor imaging with PET has become of importance not only for the improved diagnostic accuracy but also in the study of drug effects by repeat evaluation of the physiological and metabolic changes during drug-treatment (*3–5*). The evaluation of PET studies is not always straight forward due to the complexities of the biochemical pathways and the lack of quantitative data on the tracer transport and metabolism. To overcome the problem, we studied the uptake and cellular distribution of aggregates and small tissue samples to substantiate the mathematical modeling of tracer uptake, especially with regard to perturbations induced by various drugs.

Previous studies used positron emitting radionuclides in in vitro experiments (*6,7*). In these studies the advantages of using short-lived radionuclides have been demonstrated but the techniques have not been shown to be appropriate for the regular evaluation of metabolism in small tissue or cellular samples.

The multicellular spheroid tumor model offers special advantages in the use for a PET-analogue in vitro system. Spheroids have several characteristics in common with in vivo tumors; they grow in three-dimensional patterns involving cell-to-cell interactions in an extensive extracellular matrix and they develop central necrosis during growth, probably as an effect of limitation in nutrition supply (*8*, *9*). Furthermore, they constitute a cellular mass that is large enough to give adequate counts in radioactivity measurements although the sample is small enough to allow full penetration of the tracer substance.

In this study, we investigated the possibility of using ^{11}C-L-methionine as a tracer of the amino acid accumulation in spheroids, with the hope that this could be the base of further experiments towards to development of an in vitro PET analog system.

MATERIALS AND METHODS

Cell Culture

Cells from the human glioma cell line U-343 MGa $Cl_{2:6}$ (in the text called U-343 MGa) *(10)* and human colon carcinoma cell line HT-29 (ATCC, USA) were cultured in Ham's F-10 medium with 10% fetal bovine serum, L-glutamine (2nM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Flow Laboratories, Swedish AB, Stockholm, Sweden). The cultures were kept at 37°C with 5% CO_2 and the pH of the culture medium was 7.3. The medium was changed three times weekly. Monolayer cultures were trypsinized and the cells were placed in spinner flasks with 125 ml of medium. The cells were allowed to form and grow as spheroids for about 3 wk before transferring to multiwell plates and start of the experiments.

The spheroid size was measured by means of an ocular scale manipulated by a micrometer screw in an inverted Olympus microscope. The volume of each spheroid was calculated as $V = 4/3\pi \cdot (ab)^{3/2}$, where a and b were the minimum and maximum radii measured at right angles. During drug treatment, the relative volume of each spheroid was determined as the volume obtained during the treatment divided by the volume measured before treatment.

Methionine Labeled with ^{11}C and ^{14}C

The preparation of L- and D-(methyl ^{11}C)-methionine was performed as described earlier (*11*). The specific activity was 30–

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50 mCi/μmol. L-(methyl ^{14}C)-methionine and the D-(1-^{14}C)-methionine were obtained from The CEA Laboratory (Gif-Sur-Yvette, Cédex, France).

Experimental Protocol for the Study of the Methionine Accumulation

At the beginning of the experiment, the spheroids were transferred to a multiwell plate with 0.5 ml wells, with each spheroid in 50 μl culture medium placed into its own well by using Finnpipettes and polypropylene tips. Twelve to sixteen spheroids, separated into three to four groups, were used with each experimental condition.

For the preparation of the incubation medium, 30–50 MBq (~800–1400 μCi; 0.2–0.5 ml) L- or D-(methyl ^{11}C)-methionine was diluted in 3.0–3.5 ml culture medium, and 200 μl of this medium were added to each spheroid containing well. For the comparison of the effect of different specific radioactivities, L-methionine (Sigma Chemical Co.) was added to the culture medium in 48 mg/liter (314 μ*M*) concentration, which resulted in 5–10 times higher cold L-methionine concentration as compared to normal medium. The concentration of the methionine in the incubation medium varied slightly due to differences in the specific radioactivity in each delivered batch. The incubation was performed at 37°C with 5% CO_2, or in a refrigerator at 4°C for different incubation times. The washing procedure was carried out in 24-well plates by successively transferring each spheroid into three subsequent wells containing 2 ml normal culture medium. The spheroids were moved from one well to another together with 50 μl of medium by Finnpipettes and polypropylene tips. The effect of specific activity, temperature and washing time on tracer accumulation was studied.

Carbon-14-labeled L- and D-methionine were diluted in normal culture medium to give 1.0 μCi/ml concentrations. This resulted in approximately the same specific activity as in the ^{11}C experiments. The same experimental protocol was performed as in the case of ^{11}C-labeled methionine. The amount of ^{11}C activity was measured with a NaI (Tl)-crystal well detector with correction for background, radioactive decay, detector efficiency and dead-time losses. The amount of ^{14}C was measured with a liquid scintillation counter (1214 Racbeta, LKB, Wallace) after solubilization of spheroids by Biolute-S (Zinsser Analytic Ltd, Maidenhead, UK). The accumulation of labeled methionine was expressed in relative counts, obtained as the activity measured in each spheroid divided by the activity of the same volume of the incubation medium.

Analysis of the Incorporation of L-methionine into the High Molecular Fraction

After 40 or 120 min incubation and 20 min washing, the spheroids were homogenized separately in 0.5 ml normal medium using 1.0 ml syringes and fine (27G) needles. 0.5 ml normal medium was added to each homogenate to allow a better removal of the supernatant and the pellets were separated by centrifugation (1500g, 5 min). For the separation of high (>5,000) and low molecular fractions of the supernatant, Sephadex® columns (PD-10, Pharmacia Fine Chemical, Sweden) were used. The radioactivity of the pellets and the high- and the low-molecular fractions were measured separately. The sum of the pellet and the high molecular fraction was used to indicate the protein-incorporated fraction. When ^{14}C-labeled methionine was used, the fractions were treated with solubilizer before scintillation counting.

Drugs and Drug Exposures

Cycloheximide was purchased from Sigma Chemical Co. (St. Louis MO), BW A4C (*12*) was a gift from The Wellcome Research Laboratories (Beckenham, Kent, GB), AA-861 (*13*), and CV-6209 (*14*) was generously supplied from Takeda Chemical Industries (Osaka, Japan). Cycloheximide and CV-6209 were dissolved in normal culture medium and used in 10 or 30 μ*M* concentrations. BW A4C and the AA-861 were dissolved in dimethyl-sulfoxide (DMSO) and diluted in normal culture medium at a concentration of 1.0, 3.0, 10.0, or 30.0 μ*M*. (The DMSO concentration was ≤0.04%.)

The accumulation of the ^{11}C-L-methionine was measured in spheroids before and after 1 wk of treatment with 30.0 μ*M* BW A4C and 30.0 μ*M* cycloheximide. The ^{11}C-L-methionine uptake was also evaluated in spheroids exposed to 10 and 30 μ*M* AA-861 and 10 and 30 μ*M* CV-6209. The same spheroids were evaluated after 2 and 7 days of treatment. The relative counts were expressed only in relation to the pretreatment volumes. After the 7 days of treatment, the incorporation of ^{11}C-L-methionine in the high- and low-molecular fraction was measured.

For the evaluation of cell proliferation during BW A4C treatment U-343 MGa cells were plated into culture dishes (diameters: 35 mm) for monolayer growth and agarose-coated multiwell dishes for liquid overlay culture. In the overlay culture, the cells formed aggregates and were growing as spheroids. After 1 wk of culturing, 1.0, 3.0, 10.0, or 30.0 μ*M* concentrations of BW A4C were added to the monolayers and the spheroids. After 6 days of drug exposure, the cell numbers were counted in the monolayer cultures (triplicated at each concentration). The spheroids (six at each concentration) were incubated in drug- and 0.1 μCi/ml ^{3}H-thymidine-containing medium for 24 hr and the thymidine incorporation was evaluated by liquid scintillation counting.

RESULTS

Carbon-11-Methionine Accumulation in Spheroids

The accumulation of L-(methyl-^{11}C)-methionine by U-343 MGa spheroids increased linearly with time, reaching approximately 6–7 times higher concentration in the spheroid as compared to the incubation medium. The accumulation of D-methionine reached only the level of equilibration between the incubation medium and spheroids (Fig. 1A). There was a significant difference in the accumulation in the small, non-necrotic as compared to the big necrotic spheroids where the small size spheroids accumulated proportionally higher amounts of methionine (Fig. 1B).

The accumulation of the labeled methionine decreased when the concentration of cold L-methionine in the culture medium was increased 5–10-fold (Fig. 1C). The total accumulation decreased by 27%, while the protein-incorporated fraction decreased by 78%. The temperature also significantly influenced accumulation as shown in Figure 1D. When the temperature was reduced to 4°C, the L-methionine accumulation decreased by 85%. Equivalent results were found with both U-343 MGa spheroids and HT-29 spheroids in these experiments.

The washing time was demonstrated to be critical up to 5–10 min. With longer washing times, there was, however,

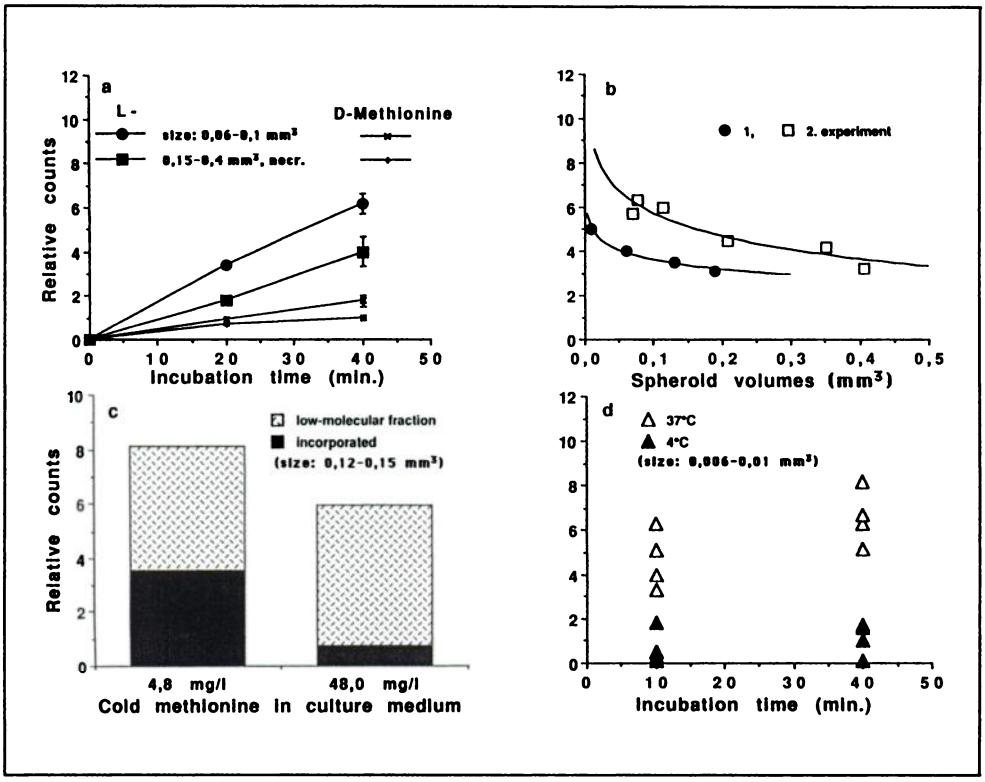

FIGURE 1. Carbon-11-methionine accumulation in U-343 MGa spheroids. (A) Accumulation of L- and D-enantiomers (means of 4 spheroids ± s.d. are demonstrated) expressed as ratio of spheroid concentration divided by concentration in incubation medium. (B) Relative uptake of L-methionine plotted against spheroid volume (incubation time: 40 min). (C) The effect of two different concentrations of L-methionine in the medium on uptake of labeled methionine (incubation time: 40 min). (D) The effect of the temperature on the incorporation of labeled L-methionine. In B and D, each point represents the relative uptake of an individual spheroid. The washing times were 5 + 5 + 10 min in each experiment.

only limited effect on the relative methionine uptake (Fig. 2A-B).

Carbon-14-Methionine Accumulation in Spheroids

Experiments using ^{14}C-labeled L- and D-methionine in HT-29 spheroids showed results very similar to those obtained in the ^{11}C-methionine studies. The concentration of the D-form in the spheroids reached a level approximately equal to that in the incubation medium, while the concentration of the L-form increased linearly with time (Fig. 3A). The accumulation kinetics showed the same characteristics as in the case of ^{11}C-methionine. The washing effect was also similar to that found with ^{11}C-labeled methionine (Fig. 3B). With L-methionine, no further washout of the tracer could be observed after about 20 min. With D-methionine, prolonged washing further decreased the concentration of methionine in the spheroid.

After 40 min incubation with ^{11}C-L-methionine, 50% of the radioactivity, and after 2 hr, 68% of the total radioactivity was found to be incorporated into the high molecular fraction (Fig. 3C).

Drug Effects on the ^{11}C-L-Methionine Accumulation in Spheroids

The effects of four different drugs on ^{11}C-L-methionine accumulation of U-343 MGa spheroids were compared. BW A4C and AA-861 are selective lipoxygenase inhibitors, CV-6209 is an etherlipid structure PAF- (platelet activating factor) antagonist and cycloheximide is a well known inhibitor of protein synthesis.

Since no data about the effects of BW A4C on tumor cell proliferation were available, the antiproliferative effects on cells cultured as monolayers as well as spheroids were investigated. U-343 MGa cells growing as monolayers were treated with BW A4C for 1 wk. There was no effect up to 3.0 μM concentration, but a strong inhibition of cell proliferation was found at 10.0 and 30.0 μM (Fig. 4A). In parallel experiments, ^{3}H-thymidine uptake of U-343 MGa spheroids decreased dose-dependently. The inhibition was significant already at 3.0 μM concentration (Fig. 4B).

Carbon-11-methionine accumulation of two spheroid groups were examined twice in one week. After the first investigation, one group was treated with 30.0 μM BW A4C for a week, and both groups of spheroids were examined after the treatment. As shown in Figure 4C the investigations using ^{11}C-L-methionine were reproducible on the same spheroids. The relation between the methionine accumulation of the treated and the untreated group did not change, indicating lack of effects on amino acid metabolism.

The effects of BW A4C and the protein synthesis inhibitor, cycloheximide, on ^{11}C-L-methionine accumulation were compared to effects on spheroid volume growth. Figure 5A shows that both compounds inhibited spheroid volume growth, but as demonstrated in Figure 5B, the effects on the methionine uptake were different. BW A4C had almost no effect on total methionine uptake, albeit the low molecular fraction was slightly inhibited and the high molecular fraction even increased. The cycloheximide treatment on the other hand induced a very strong inhi-

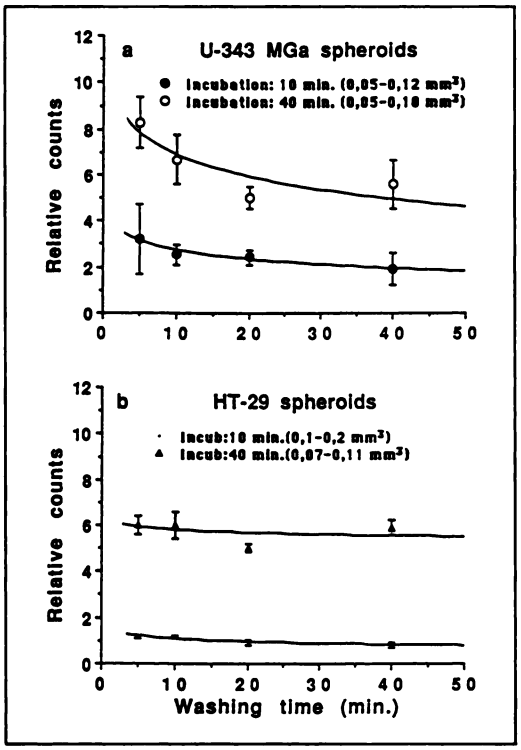

FIGURE 2. The effect of washing time on the measured relative L-methionine uptake in U-343 MGa glioma (A) and HT-29 colon cancer (B) spheroids. The points demonstrate the mean of four spheroids ± s.d.

bition in the methionine incorporation into the high molecular fraction.

In the cases of AA-861 and CV-6209 treatment, the ^{11}C-L-methionine uptake was normalized to the spheroid volumes measured at the beginning of the experiments. In the spheroids treated with AA-861, no effects on the total incorporated methionine could be found after 2 and 7 days treatment (Fig. 6A-B). A slight overall increase in the uptake in the untreated spheroids and spheroids treated with AA-861 was observed. With CV-6209, a clear reduction in the methionine incorporation was found, especially after 7 days of treatment (Fig. 6C-D).

The fraction of protein-incorporated methionine was close to 50% in the controls. There was a tendency towards an increase of the incorporated fraction in the spheroids treated with AA-861, while the low-molecular fraction decreased at 30.0 μM concentration (Figure 6B). In the group treated with CV-6209, the fraction of protein incorporated methionine was significantly reduced (Fig. 6D).

DISCUSSION

This paper discusses some methodologic aspects of the use of short-lived positron-emitting radionuclides for the in vitro study of metabolism in multicellular spheroids. It seems possible to use this technique for the evaluation of metabolism and drug testing. The accumulation kinetics of amino acids and the pathways of protein synthesis are

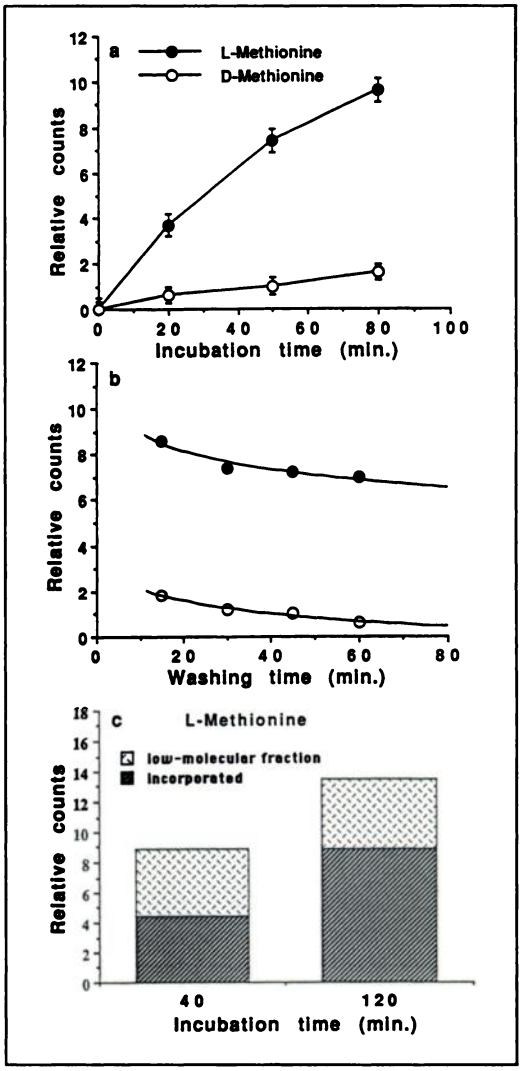

FIGURE 3. Carbon-14-methionine accumulation in HT-29 spheroids. (A) Accumulations of L- and D- enantiomers related to incubation time (means of 4 spheroids ± s.d.). (B) The effect of washing time on the relative uptake of ^{14}C-L- and D-methionine (incubation time: 40 min) (means of three spheroids). (C) Demonstration of the L-methionine incorporation into the high molecular and low molecular fractions (washing time: 5 + 5 + 10 min) (means of four spheroids).

well-described based on investigations in different in vitro and in vivo systems (*15–17*). Our experiments have demonstrated that the accumulation of methionine can be studied in multicellular spheroids using ^{11}C-L-methionine. Significant differences in the accumulation of the two stereoisomers of methionine in the spheroids were observed: The activity of D-methionine only equilibrated with the medium, while the activity of the L-form increased linearly with time, in which after a 40-min incubation time about a 6–7 times higher concentration was reached in the spheroids than that measured in the incubation medium. This degree of accumulation and the stereospecificity correspond well to the in vivo findings using PET (*18–21*). With this incubation time, approxi-

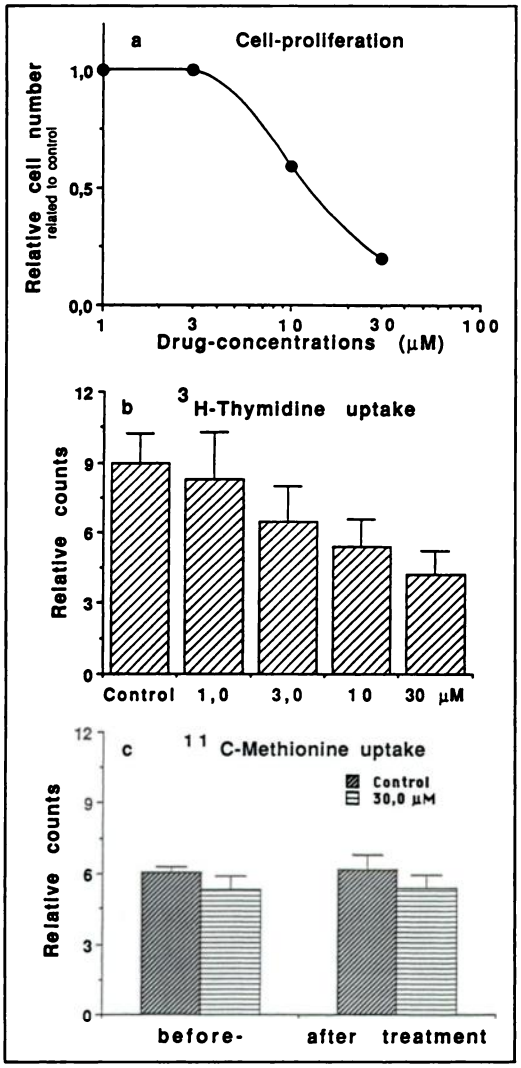

FIGURE 4. The effect of BW A4C treatment on proliferation of U-343 MGa cells, cultured as monolayers (A), and on the ^{3}H-thymidine (B) and ^{11}C-methionine (C) uptake in U-343 MGa spheroids. The monolayer cultures were triplicated and means of 4 spheroids ± s.d. are indicated.

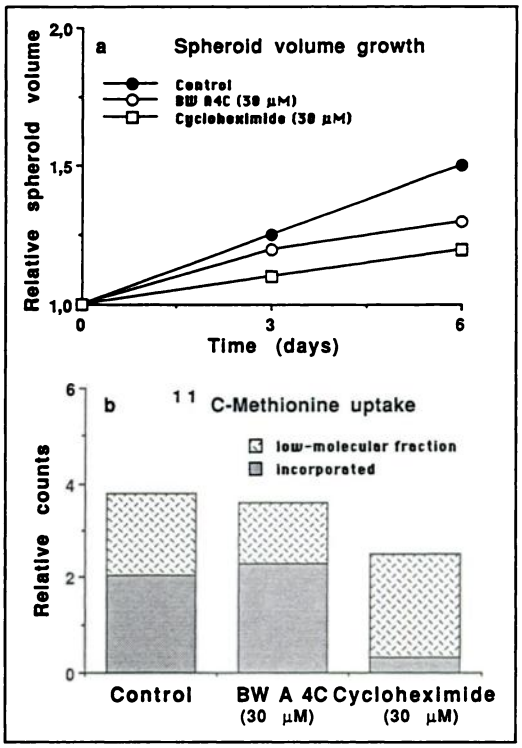

FIGURE 5. Drug-effects on spheroid volume growth (A) and on ^{11}C-methionine uptake (B). Means of four spheroids are demonstrated. Standard deviations were less than 30% of the means.

mately 50% of the accumulated ^{11}C-L-methionine was incorporated into the high molecular fraction (*22*).

The significance of the accumulation of methionine in brain tumors as observed with PET has been discussed (*23*). Among the different factors responsible for this accumulation, transport phenomena as well as incorporation into proteins have attained the most interest. It is clear that some transport phenomena, such as facilitated transport across the blood-brain-barrier (BBB), are stereoselective (*24*). Furthermore, protein incorporation is highly stereoselective. D-methionine is not incorporated into proteins. The fact that D-methionine reaches a concentration in spheroids equal to that in the incubation medium indicates that transport across the cell membrane is not a limiting factor. Transport across the BBB is saturable and can be decreased by competition with other amino acids (*23*). This transport is not directly energy-dependent but could conceivably still be affected by temperature. Transport across the cell membrane is attributed to the A-transport system, which is energy-dependent and should thus be affected by temperature. A lowering of the temperature will affect both incorporation into proteins and energy-dependent transport mechanisms. Thus, the very pronounced decrease in methionine uptake observed at 4°C could be explained by the combination of the two factors.

The limited decrease in tissue radioactivity as a whole observed when large amounts of nonradioactive methionine were added to the medium could be explained by the fact that a large fraction of the labeled methionine remains unmetabolized but trapped in tissue, or that the label is transferred to other low-molecular weight substances. Protein-incorporated methionine decreased nearly proportionally to the increase of nonradioactive methionine concentration in the medium, but the pool of low-molecular substances was relatively unaffected. The hypothesis of two intracellular pools with different characteristics, one with free methionine or other low-molecular metabolites and one with protein-incorporated methionine, was further supported from the experiments with added cycloheximide. The protein-incorporated fraction was strongly inhibited by cycloheximide, whereas the pool of low-molecular substances was unaffected. One aspect of interest was that the pool of low-molecular substances could not be washed away in the time span of the study.

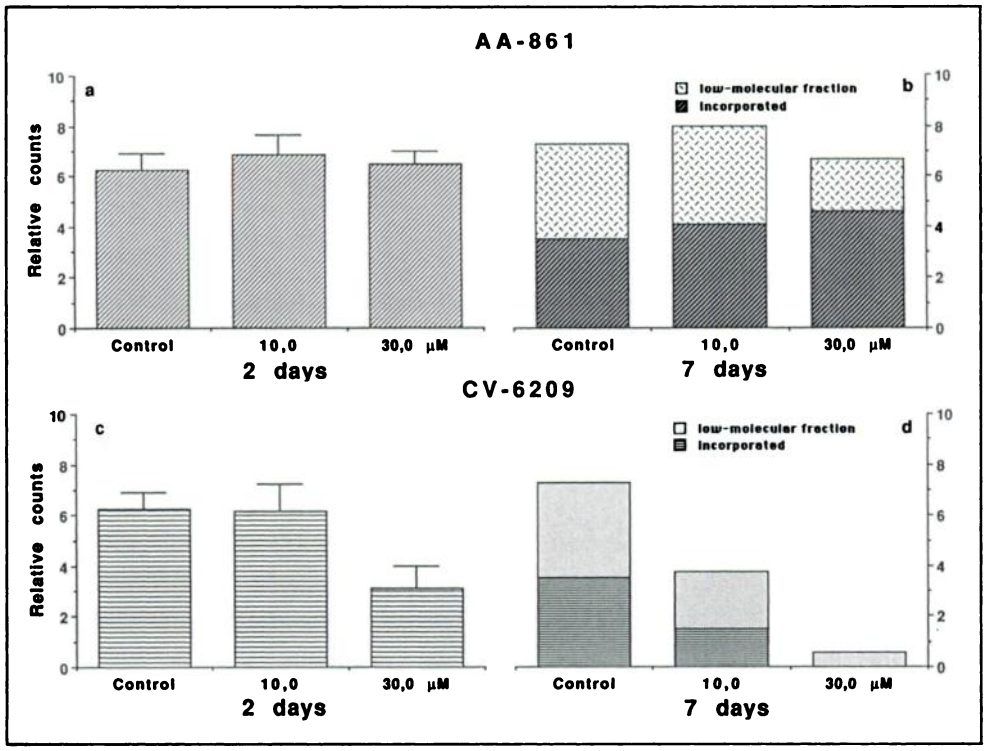

FIGURE 6. The effect of the drugs AA-861 and CV-6209 on methionine uptake, measured in the same spheroids at 2 (A, C) and 7 (B, D) days after addition of the drugs. The fractional incorporation of methionine into high-molecular and low-molecular fraction were determined in the same spheroids on the seventh day of treatment (B, D).

It was not possible to separate the influence of transport mechanisms, intracellular storage of unmetabolized methionine, and incorporation into proteins. Furthermore, methionine is a methyl donor to other metabolic processes, and with ^{11}C-methionine labeled in the methyl-group, this factor will add to the binding of the label in the tissue. Therefore, we cannot assume that the technique only measures true protein synthesis. Studies with added drugs demonstrate, however, that the ^{11}C-methionine studies do demonstrate a functional response, which covers the sum of different metabolic processes, of which protein synthesis probably constitutes the greater part. In view of the above, we therefore prefer to denote the measurements obtained in vitro or in vivo with ^{11}C-L-methionine: methionine uptake.

The drugs used in the study demonstrated different effects on glioma cells. Cycloheximide (*25*) and CV-6209 (*26*) as well as the lipoxygenase inhibitors AA-861 (*27*) and, as we demonstrated, BW A4C all had strong antiproliferative properties as measured in effects on cell proliferation or thymidine incorporation. Their effects on L-methionine uptake were, however, different. Our experiments demonstrated that 30.0 μM of the protein synthesis inhibitor cycloheximide caused a significant decrease in methionine uptake, especially related to the protein-incorporated fraction, while the low-molecular fraction even increased slightly. Treatment with 30.0 μM BW A4C did not have a significant effect on methionine accumulation of spheroids. The same dose of BW A4C caused a very strong decrease in cell proliferation of monolayers and in ^{3}H-thymidine incorporation of spheroids. In these experiments, when evaluating the uptake of labeled methionine in spheroids, we corrected for the actual spheroid volumes measured at the time of the investigations. The volume growth of the treated spheroids was, however, inhibited during the treatments due to decreases of cell proliferation and/or shrinkage of spheroid tissue. We have to consider that by this calculation a possible shrinkage might cause an increase in measured uptake. With AA-861 and CV-6209, we related the methionine uptakes, obtained during the treatments, to the spheroid volumes measured at the beginning of the experiments. This type of calculation probably better reflects general antitumoral drug effect. AA-861, similarly to BW A4C did not decrease the ^{11}C-L-methionine uptake, and after one week treatment the protein incorporated fraction even increased slightly, as compared to the control.

We can only speculate on why the lipoxygenase inhibitors BW A4C and AA-861 were effective in the inhibition of cell proliferation of monolayers and in ^{3}H-thymidine incorporation of spheroids, while they did not show any inhibitory effect on L-methionine uptake. Lipoxygenase products of arachidonic acid cascade are known to increase edema formation by increasing the penetration of fluids into the tissues (*28*). The slight shift in the relation to the low- to high-molecular fraction of methionine uptake could thus be explained by the decreased water content of the tissue. Since BW A4C and AA-861 are inhibitors of the 5-lipoxygenase, these drugs can probably decrease the penetration of substances, e.g., nutrients, into tissue and thereby create unfavorable conditions for the inner cell layers.

The good antiproliferative effect and the lack of effect on methionine uptake also points to effects on specific mechanisms involved in DNA synthesis. The fact that the same observations were made with two different lipoxygenase inhibitors with different chemical structures, indicates that this effect could be directly related to the lipoxygenase system. How this is exerted is presently not known. Lipoxygenase will generate not only leukotrienes but also intermediates such as 5-HETE and lipoxins, which could mediate the effects. It is also possible that lipoxygenase inhibition can stimulate the cyclo-oxygenase route of the arachidonic acid cascade, resulting in the enhanced production of toxic prostaglandins. These speculations certainly do not answer the question regarding the lack of inhibitory effect for methionine uptake, but may outline the difficulties in the interpretation of the in vivo PET results and emphasize the importance of a PET analog in vitro system. It is conceivable that some types of treatment will affect proliferation without altering the protein synthesis and that in these instances PET with ^{11}C-methionine will not adequately indicate the effect. Such treatments probably include radiation therapy and cytostatics, which primarily exert their effects on DNA. It is likely that also in the case of lipoxygenase inhibitors, ^{11}C-methionine is not the ideal tool for the recording of antitumoral effects. In these cases the use of ^{11}C-thymidine would be preferred.

With the etherlipid-structure PAF-antagonist CV-6209, a very pronounced decrease in methionine uptake was observed. This substance has been shown to be a very potent inhibitor of glioma cell proliferation (*26*). The antiproliferative properties of ether-phospholipids have been ascribed to the fact that they are inserted in cell membranes and thus change membrane properties (*29*). These drugs may be assumed to affect cells in a more general sense and it is likely that PET with ^{11}C-methionine can adequately be used to monitor treatment response in clinical trials.

The present study describes a rapid, repetitive in vitro technique for the evaluation of cellular metabolism using substrates labeled with short-lived positron-emitting radionuclides. This can be useful in the evaluation of drug effects on amino acid accumulation, either in the preclinical screening, before a drug is applied in vivo, or in parallel with in vivo investigations. We are presently extending this technique to biopsy samples and small surgical tissue specimens.

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SELF-STUDY TEST

Radiobiology and Radiation Protection

Questions are taken from the *Nuclear Medicine Self-Study Program I,* published by The Society of Nuclear Medicine

DIRECTIONS

The following items consist of a heading followed by numbered options related to that heading. Select those options you think are true and those that you think are false. Answers may be found on page 2280.

The anticipated effects on an individual of a whole-body radiation dose of 100 rads include:

1. a significant reduction in immune responsiveness
2. permanent sterility
3. a lifetime risk of about 1% for radiation-induced fatal cancers
4. a high likelihood of genetic effects in his or her children
5. epilation and bleeding of gums

True statements concerning nonstochastic effects of ionizing radiation include:

6. The severity of the effect varies with dose.
7. The probability of the effect varies with dose.
8. There often is a threshold dose.
9. The aim of radiation protection should be to prevent these effects.
10. They are limited by cell killing.

The genetically significant dose (GSD)

11. is the dose of radiation each person receives from birth to death.
12. is the dose of radiation that can be shown to have led to a genetic death.
13. from medical exposure in the U.S. is approximately equal to that from background sources.
14. is an index of the presumed genetic impact of radiation exposure to the population.

True statements concerning the genetic "doubling dose" for radiation-induced genetic abnormalities include:

15. It is the amount of radiation that would be expected to add as many new mutations as occur spontaneously.
16. The higher the doubling dose, the greater the risk of *mutations* for a given amount of exposure.
17. A doubling dose administered to a population would produce twice the spontaneous number of mutations in the next generation.
18. It is the reciprocal of the relative mutation risk.
19. The BEIR 1980 estimate of a doubling dose of 50–250 rads was obtained from human epidemiologic studies.

True statements concerning the genetic effects of radiation include:

20. Mutations are usually harmful.
21. Genetic effects observed in the progeny of the A-bomb survivors provide the best estimate of human risk.
22. They appear to depend very little on the stage of germ cell development at irradiation.
23. They are independent of the rate of delivery of the radiation.
24. Their likelihood decreases as the time interval between irradiation and conception increases.

(continued on p. 2323)