Anti-Cancer Drugs, 3, pp. 519–523

Selective uptake of the somatostatin analog RC-160 across the blood–brain tumor barrier of mice with KHT sarcomas

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The development of somatostatin analogs with anti-tumor effects has raised hopes for their use in various cancers and tumors of the central nervous system. However, for many therapeutic agents, access to normal brain is retarded by the blood–brain barrier (BBB) and to tumor tissues by a blood–brain tumor barrier (BBTB). We examined the ability of RC-160, a somatostatin analog with known anti-tumor activity, to cross the normal BBB and the BBTB in mice with brain sarcomas. In comparison with the normal BBB, the BBTB was about 10 times more permeable to the vascular marker albumin (radioactively labeled with ^{99m}Tc), but the BBTB still represents a substantial barrier. By contrast, the entry rate of RC-160, radioactively labeled with ^{125}I, into brain sarcomas was 60 times higher than into normal brain tissue; more than 1% of the RC-160 injected i.v. was taken up by each gram of brain tumor. These results show that a brain tumor can selectively accumulate the potentially therapeutic agent RC-160.

Key words: Blood–brain barrier, RC-160, sarcoma, somatostatin.

Introduction

Tumors of the central nervous system (CNS), both primary and metastatic, continue to offer one of the greatest challenges to the development of therapeutic agents. Progress in the development of effective chemotherapeutic agents active against peripheral cancers cannot usually be directly applied to tumors of the CNS because the blood–brain barrier (BBB), comprised primarily of the capillary bed, often remains intact to some degree.[1]

Nevertheless, the development of somatostatin analogs with powerful effects against a variety of cancers,[2,3] binding sites on brain tumor cells,[4] an apparently selective uptake by intracranial tumors[5] and limited permeability across the normal BBB[6] has raised the hope that these agents might mors[5] and a limited permeability across the normal BBB[6] has raised the hope that these agents might be able to cross the blood–brain tumor barrier (BBTB). Here, we assess the permeability of the BBTB to the therapeutic somatostatin analog RC-160 and the vascular marker albumin in comparison with the normal BBB.

Materials and methods

Peptide synthesis and radioactive labeling

The somatostatin analog RC-160 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2) was synthesized by solid phase methods in our laboratory and purified by HPLC.[7] RC-160 was radioiodinated by mixing

This work was supported by the Department of Veterans Affairs.

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5 μg of peptide in 5 μl of 0.01 M acetic acid with 40 μl of 0.5 M sodium phosphate buffer, 1 mCi of ^{125}I and chloramine T. The reaction was stopped 30 s later by adding cysteine in 0.5 ml of 0.5 M phosphate buffer. The labeled RC-160 (I-RC) was purified by HPLC with a C_{18} column (W-Porex 5C18, Rancho Palos Verdes, CA). Kits (Medi + Physics, Paramus, NJ) were used to label albumin with ^{99m}Tc (Tc-Alb).

Implantation of sarcoma cells

KHT malignant sarcoma cells were grown in RPMI 1640 medium supplemented with 10% colostrum-free bovine serum, antibiotics and antimycotics. Cultures were incubated in 5% CO_2 in air at 37°C. Cell populations in the exponential growth phase having a viability of greater than 90% (as determined by ability to exclude trypan blue) were used for inoculation.

KHT malignant sarcoma cells were implanted as previously described[8] into 2–3 month old C3H/HEN MTV-negative female mice (Simonsen Laboratories, Gilroy, CA). Mice were anesthetized with methoxyflurane (Pitman Moore, Denver, CO). A midline incision was made over the anterior aspect of the cranium and the scalp retracted to the right. A guarded 26 gauge needle was used to drill a hole in the skull 3 mm to the right of midline, just anterior to the coronal suture, and 3–4 mm deep. A Hamilton syringe (Reno, NV) was used to inject 10 μl of 0.09% sterile sodium chloride containing about 10^5 viable KHT sarcoma cells. The scalp was closed with surgical skin staples. Control mice were treated identically, except that their intracranial injections contained no tumor cells.

Determination of entry rates and vascular spaces

Unidirectional influx rates (K_i) of I-RC and Tc-Alb from blood into normal brain and brain tumor tissues were determined by a previously described graphical method[9,10] 14 days after the intracranial injection. At 60 min after an i.p. injection of trypan blue (200 μl of 50 mg/ml), mice anesthetized with urethane received a bolus injection into the jugular vein of 200 μl of lactated Ringer's solution with 1% bovine serum albumin containing 10^6 c.p.m. of Tc-Alb and 10^6 c.p.m. of I-RC. Another group of tumor-bearing mice received an additional 100 μg of unlabeled RC-160 in their i.v. injections.

Blood was collected from a cut in the carotid artery between 1 and 16 min after the i.v. injection and the brains immediately removed. In mice injected with tumor cells, discrete tumors were easily identified by being stained with trypan blue, dissected free of surrounding normal tissue and weighed. Portions of normal cerebral cortex from the tumor-free side contralateral to the intracranial injection site were also collected and weighed. In control mice, portions of normal cerebral cortex were removed from the side ipsilateral to the site of injection.

The levels of ^{99m}Tc and ^{125}I were determined in a gamma counter and the results expressed as a ratio (R) of the (c.p.m./g of tissue)/(c.p.m./ml arterial serum) (in ml/g). This was plotted against the exposure time (T) which was calculated as described[10] by dividing the integral for the time–blood radioactivity level to time t by the blood radioactivity level at time t. The linear portion of this plot was taken to be the unidirectional influx rate, or K_i (in units of ml/gmin) and its y intercept the apparent volume of distribution, or V_i (in units of ml/g). In the absence of a correlation between R and T, R was taken to represent the distribution or vascular space.

The percent of i.v. injection per gram of brain was corrected for vascular space by using the V_i of Tc-Alb for the tumor group and the mean of R for the normal brain.

Statistical analysis

Means are reported with their standard errors and were compared by analysis of variance (ANOVA) followed by Duncan's multiple range test. Regression lines were computed by the least squares method and were compared with the P1R program from the BMDP statistical software package (University of California, Berkeley, CA).

Results

The accumulation of I-RC by brain tumor (Figure 1) with a K_i of 5.90×10^{-3} ml/g min was greater than any other rate of accumulation measured, including that of Tc-Alb by brain tumor [$F(2,16) = 40.7$, $p < 0.000005$) and that of I-RC by contralateral normal brain tissue before [$F(2,14) = 40.3$, $p < 0.000005$] or after [$F(2,14) = 42.3$, $p < 0.000005$) the correction for vascular space and leakage of the capillary bed as measured by Tc-Alb.

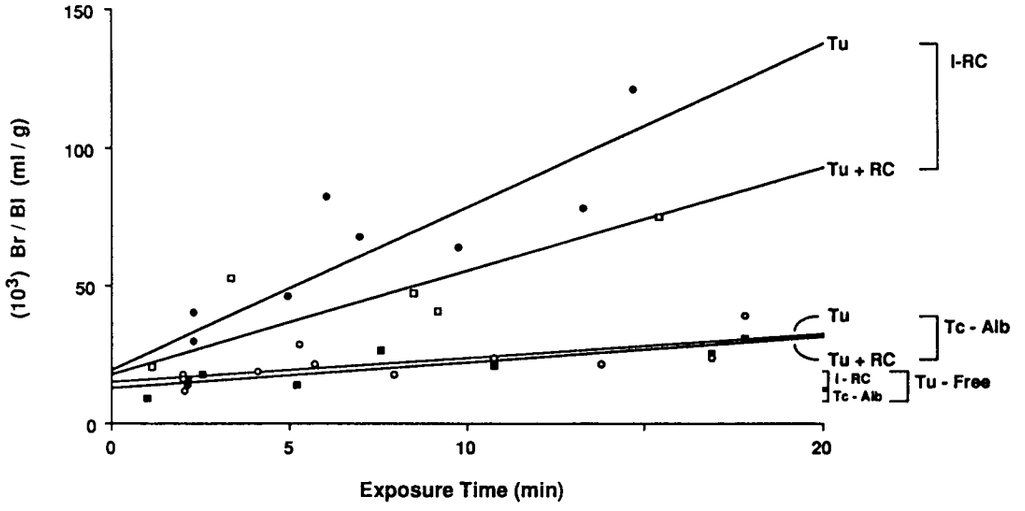

Figure 1. Accumulation of I-RC and Tc-Alb by brain tumors (Tu). The effect of inclusion in the i.v. injection of unlabeled RC-160 (Tu + RC) on accumulation is also shown. The range for accumulation in tumor-free brain tissue (Tu-Free) is also indicated.

Inclusion in the i.v. injection of unlabeled RC-160 decreased the K_i for I-RC accumulation by brain tissue to 3.73×10^{-3} ml/g min [$F(2,12) = 3.9$, $p < 0.05$].

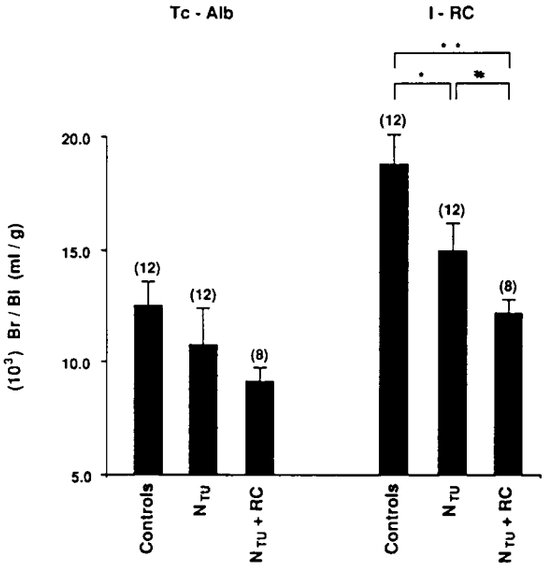

Figure 2. Uptake of I-RC and Tc-Alb by the three groups of tumor-free brain tissue: controls, normal brain tissue from brain tumor bearing mice that did (N_{TU} + RC) or did not (N_{TU}) have unlabeled RC-160 included in their i.v. injection. The number in parentheses above the SE bar is the n value. $^{**}p < 0.0005$; $^{*}p < 0.05$; $\#0.05 < p < 0.1$.

The accumulation of Tc-Alb by brain tumors (Figure 1) with a K_i of 0.836×10^{-4} ml/g min was greater than that for contralateral normal brain tissue [$F(2,18) = 16.7, p < 0.0001$]. Inclusion in the i.v. injection of unlabeled RC-160 had no significant effect on Tc-Alb accumulation.

Accumulation of I-RC and Tc-Alb by normal brain tissue is indicated in Figure 1 and shown in detail on an expanded scale in Figure 2. For these tissues, the relationship between R and T was not statistically significant and so R primarily reflects V_i. A statistically significant difference occurred among these tissues (Figure 2) for uptake of I-RC before [$F(2,30) = 8.37$, $p < 0.005$] and after [$F(2,30) = 5.43$, $p < 0.05$] correction for the vascular space as measured by Tc-Alb. No statistically significant difference was found among these groups for Tc-Alb uptake. The uptake of I-RC by control brain tissue was greater than the uptake of Tc-Alb [$F(1,10) = 13.1$, $p < 0.005$]. The percent of the injection of I-RC in tumor was over 8 times greater than in the contralateral tumor-free brain tissue [$F(1,22) = 36.9$, $p < 0.001$], see Figure 3.

Discussion

The results show that I-RC is selectively taken up by KHT sarcoma brain tumors. The entry rate as measured by the K_i of I-RC by brain tumor was about 60 times greater than that previously

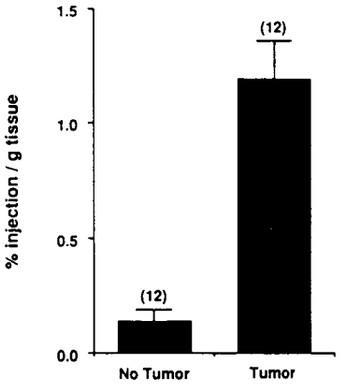

Figure 3. Percent of injection entering a gram of brain tumor or normal brain tissue from a tumor-bearing animal. Values are collapsed across time. $p < 0.001$.

measured for normal brain tissue[6] and about 7 times greater than the entry rate of Tc-Alb into these tumors.

This enhanced entry of I-RC into the brain tumors can be partially explained by the enhanced leakiness of the BBTB in comparison with the normal BBB. The greater leakiness of the BBTB was shown by the staining of the tumors by the trypan blue that was administered i.p. and by a K_i for Tc-Alb entry into the tumors about 10 times greater than usually measured for vascular markers.[9,11] This is consistent with previous findings on non-glial brain tumors.[1,12] Further support of the leaky nature of the BBTB is provided by our observation that the permeability of the brain tumors to I-RC is 7 times greater than to Tc-Alb; this is similar to what would be predicted for the two compounds based on a comparison of the inverse square roots of their molecular weights, a useful predictor for entry rates for porous membranes.[13] Nevertheless, a substantial barrier remained at the BBTB, with a K_i for Tc-Alb still only one-third that of the blood–testis barrier.[14]

Increased leakiness cannot, however, completely explain the enhanced uptake of I-RC for two reasons. First, in normal brain, the K_i of circulating I-RC is low, only about 1.5 times greater than the K_i for vascular markers such as Tc-Alb.[6] The relatively low K_i for circulating I-RC is thought to be due to a factor found in the circulation, since the K_i increased by more than 200 times when serum-free perfusions of the brain were used. A similar situation has been found for vincristine and vinblastine.[15] This circulating factor binding I-RC is likely to be the serum protein that binds endogenous somatostatins.[16] Such binding would tend to negate the difference in molecular weight between I-RC and Tc-Alb.

Second, the inhibition of entry into tumor tissue of I-RC but not of Tc-Alb by unlabeled RC-160 cannot be explained on the basis of a leaky membrane. A decrease in the size of pores could disproportionately affect the larger Tc-Alb, and a decrease in the number of pores would affect both I-RC and Tc-Alb equally.[17] Self-inhibition indicates a saturable component to the accumulation process. Although I-RC can be selectively pumped out of the CNS, entry into the brain is by a non-saturable process in CD-1 mice and Sprague-Dawley rats.[6] It is likely, therefore, that the site of self-inhibition is at the level of previously described receptors on the sarcoma cell that can bind I-RC[18] or perhaps that the BBTB has developed a transport mechanism for I-RC. These results are also consistent with the ability of RC-160 to prolong survival in mice with intracranial KHT sarcomas.[18]

Comparable considerations led Wienhard *et al.*[19] to similarly conclude that the enhanced entry of the amino acid fluorotyrosine into brain tumors was independent of an accompanying increase in BBTB permeability. In that study, the increase in the K_i for fluorotyrosine was twice as great in brain tumors as in contralateral normal tissue, while the increase for the vascular marker was 4-fold. A 10-fold increase in the K_i for brain tumors in comparison to tumor-free brain tissue was found for meglumine iothalamate while increases as high as 50-fold were found only focally in some tumors.[20] The 60-fold increase found here for I-RC, therefore, would seem to represent a very high selectivity by brain tumor tissue. Similarly, an 8-fold increase in the accumulation of I-RC by tumors in comparison with contralateral normal cerebral cortex exceeds the 3- to 6-fold increase found for radioactive palmitate, an agent that may be useful in imaging brain tumors.[21]

Uptake of Tc-Alb and I-RC by normal brain tissue was much lower than that of brain tumor tissues. The inability to accurately determine K_i's probably reflects this low uptake and the shorter study times than previously used.[6] The R values for Tc-Alb in normal brain tissue are consistent with the size of the vascular space for brain tissue. The larger R values for I-RC, however, indicate passage across the BBB, and are consistent with previous studies showing passage[6] and effects on analgesia through a CNS site.[22] This uptake of I-RC by normal brain tissue, either with or without correction for the vascular space by subtraction of

the values for Tc-Alb, was less in mice with brain tumors. The enhanced uptake of I-RC, by extrapolation, might suggest an enhanced uptake of endogenous circulating somatostatins by brain tumor tissue. The decreased uptake in normal cerebral cortical tissue of mice with tumors in the contralateral side was decreased in comparison with control mice without tumors in either side. This raises the possibility of a linkage of uptake rates between the two hemispheres.

In conclusion, the BBTB of the KHT sarcoma was about 10 times more leaky than the normal BBB as measured by Tc-Alb. The entry rate of the potential therapeutic agent RC-160 was about 60 times greater than in normal brain and cannot be fully explained by the increased leakiness of the BBTB. This selective increase in the uptake of RC-160 is likely to be due in part to the ability of the KHT sarcoma cell to bind I-RC or perhaps to the selective uptake of I-RC by the BBTB. These findings raise the hope for the development of a new treatment for brain tumors based on somatostatin analogs.[3,23]

Acknowledgment

We wish to thank Melita B Fasold for help with manuscript and graphics preparation.

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(Received 13 July 1992; accepted 12 August 1992)