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Hyaluronidase pretreatment produces selective melphalan enrichment in malignant melanoma implanted in nude mice

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Abstract Preclinical and clinical observations suggest that the administration of hyaluronidase (Hyase) shortly before that of chemotherapy increases the access and, thus, the effectiveness of aniticancer drugs in tumors. To examine this hypothesis as well as the selectivity of such a therapeutic approach potentially beneficial in isolated limb perfusion, the Hyase-induced distribution of melphalan was measured in tumor-bearing nude mice with respect to the mode of drug administration using RP-18 ion-pair high-performance liquid chromatography (HPLC) with fluorimetric detection. Melphalan alone (50 μmol/kg) or a combination of melphalan (50 μmol/kg) and Hyase (100,000 IU/kg) was injected either i.p. or s.c. in the vicinity of the tumors. The s.c. melphalan injection caused a 4-fold rise in melphalan concentration (59 μ*M*) in the tumors as compared with i.p. application (15 μ*M*). Only minor effects were observed with respect to the route of melphalan application on its distribution in other tissues (ca. 13 μ*M* in plasma, 15 μ*M* in muscle, 30 μ*M* in the liver, 26 μ*M* in the kidney, and 21 μ*M* in the testicle). Irrespective of the route of Hyase coadministration, the enzyme increased the concentration of i.p. injected melphalan in all tissues to ca. 20 μ*M* in the tumor, 15 μ*M* in plasma, 27 μ*M* in muscle, 40 μ*M* in the liver, 29 μ*M* in the kidney, and 28 μ*M* in the testicle. In contrast, s.c. injected melphalan was selectively accumulated by the tumors after both s.c. and i.p. Hyase administration (462 and 388 μ*M*, respectively). Melphalan enrichment in the tumors was higher (16- to 32-fold higher than in the other tissues) after i.p. administration of Hyase since, in contrast to s.c. injection of the enzyme, its i.p. administration caused a decrease in the concentration of the cytostatic in all other tissues as compared with the s.c. administration of melphalan alone.

Key words Hyaluronidase · Melphalan · Malignant melanoma · Drug distribution

Introduction

A widely neglected reason for the unsatisfactory outcome of cancer chemotherapy has recently been seen in the insufficient penetration of cytostatics into solid tumors. Besides the elevated intratumoral hydrostatic pressure, which counteracts drug penetration, natural barriers such as the basal laminae and the tumor stroma impair a uniform spreading of the chemotherapeutics within the neoplasm [19]. As a major component of the extracellular matrix, the hyaluronic acids are vital in controlling diffusion processes [24]. Decker et al. [13] have reported that hyaluronic acid is also synthesized in tumors either by fibroblasts after paracrine stimulation or by the tumor cells themselves. As a consequence, elevated levels of hyaluronic acid have been observed not only in mesotheliomas, where they are diagnostically useful as tumor markers, but also in the serum of breast cancer or malignant melanoma patients suffering from metastasized or large tumors [11].

These observations were the rationale for the addition of the hyaluronic-acid-degrading enzyme hyaluronidase to conventional chemotherapy regimens [2, 3] or for its combination with radiochemotherapy [32]. Although these experimental therapies were beneficial to most patients, with a few exceptions [4] these studies were pilot in nature. The clinically observed recoveries can be explained by a hypothetical mechanism of action of this experimental therapy: the degradation of the extracellular matrix by hyaluronidase reduces the edemas around and within the

Dedicated to Prof. Dr. Dr. Walter Schunack on the occasion of his 60th birthday

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tumors, thus providing higher effective concentrations of the coinjected chemotherapeutics in the tumor area [3, 5, 36].

However, preclinical investigations on the potentiation of the antitumor activity of the coinjected chemotherapeutics by hyaluronidase, which would provide an experimental basis for the clinical application of hyaluronidase, are rare both in in vitro experiments [5, 20, 23, 25, 31] and in animal models [5, 29, 30, 33, 36] as well as being contradictory in part. Even scarcer are the data on hyaluronidase pharmacokinetics [14, 21, 40] or studies on the resorption and penetration of cytostatics into tumors in combination with hyaluronidase [12, 20]. Czejka et al. [12] concluded from the plasma levels of 5-fluorouracil (5-FU) achieved with and without intraarterial injection of hyaluronidase in the same patient that hyaluronidase improves the distribution of 5-FU in the tumor, whereas Kohno et al. [20] observed a hyaluronidase-induced enhancement of doxorubicin in multicellular tumor spheroids using fluorescence microscopy. To our knowledge, no report in the literature has focused on possible hyaluronidase-induced differences in the distribution of chemotherapeutics in tumors and other tissues.

Very recently we have reported that regional combination chemotherapy with vinblastine and hyaluronidase is highly effective in curing human malignant melanomas in nude mice [35, 36]. In contrast to systemic chemotherapy, regional chemotherapy of the malignant melanoma, which allows a markedly higher dose of the drugs, leads to long-persisting remissions or even to cures [1]. In patients with metastasized melanoma confined to an extremity, hyperthermic isolated limb perfusion with melphalan [38] is the preferred therapeutic procedure.

In this context we were interested in investigating whether a combination of hyaluronidase with melphalan would produce elevated drug levels in malignant melanomas, thus making isolated limb perfusion more effective. In this paper we report on the hyaluronidase-induced differences in the distribution of high-dose melphalan to the tumor, plasma, and muscle as well as the liver, kidney, and testicles of nude mice as possible targets of melphalan toxicity [28] with respect to the mode of drug application.

Materials and methods

Chemicals

All chemicals were of analytical grade. NaCl, KCl, $Na_2HPO_4 \cdot 2H_2O$, $NaH_2PO_4 \cdot H_2O$, KH_2PO_4, $NaHCO_3$, HCl, and H_3PO_4 were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was purchased from Serva (Heidelberg, Germany). Methanol was of high-performance liquid chromatography (HPLC) grade (Baker, Groß-Gerau, Germany), and Millipore-filtered water was used throughout all experiments.

Drugs

Melphalan was purchased from Sigma (München, Germany). Hyaluronidase from bovine testes was kindly provided in the highly purified lyophilized form as Neopermease in vials containing 200,000 IU of the enzyme (specific activity approx. 50,000 IU/mg protein), with gelatin (25 mg) serving as the carrier, by Sanabo GmbH (Vienna, Austria).

Tumor cell line and culture conditions

The human malignant melanoma cell line SK-MEL-2 was purchased from the American Type Culture Collection (Rockeville, Md., USA). Cell banking and quality control were performed according to the "seed stock concept" [18]. The cells were maintained in Eagle's minimum essential medium (EMEM) containing L-glutamine (Sigma, München, Germany), 2.2 g $NaHCO_3$/l, 110 mg sodium pyruvate/l (Sigma, München, Germany), and 10% fetal calf serum (FCS; Gibco, Eggenstein, Germany). The cells were cultured in a water-saturated atmosphere of 95% air and 5% CO_2 at 37° C in 75-cm^2 culture flasks (Costar, Tecnomara, Fernwald, Germany) and were serially passaged following trypsinization using trypsin (0.05%) /ethylenediaminetetraacetic acid (EDTA, 0.02%; Boehringer, Mannheim, Germany). For tumor cell implantation into nude mice the cells were harvested mechanically in passage 36 from origin, spun down at 100 *g* for 10 min, and washed twice with serum-free EMEM. The cell concentration was adjusted to approx. 10^7 cells/ml of serum-free EMEM. Solid melanomas were established in NMRI *nu/nu* mice by s.c. injection of about 2–3 million tumor cells/animal.

Animal and housing conditions

NMRI *nu/nu* mice were randomly bred in the authors' nude mouse laboratory under specific pathogen-free conditions at 26° C, 70% relative humidity, and a 12-h/12-h light/dark cycle [37]. Nude mice were fed ad libitum with combined breeding-maintenance diet 1434 (Altromin, Lage, Germany) and water containing 1.3 g potassium sorbate/l (Merck, Darmstadt, Germany) and 2 g chloramphenicol/l (Sigma, München, Germany); the pH was adjusted to 2.5 with HCl [17]. All animals were housed in macrolon cages of size III (Ehret, Emmendigen, Germany).

Tumor transplantation and treatment

Solid SK-MEL-2 tumors were established in 9- to 10-week-old NMRI *nu/nu* mice by s.c. injection of cultured cells. For serial passage the tumors were cut into 2-mm^3 pieces and transplanted s.c. into the region of the thoracic mammary fat pad with a trocar. The influence of hyaluronidase on melphalan distribution was studied in male animals (mean body weight 28.6 $\pm$ 2.9 g) bearing solid human SK-MEL-2 melanomas in passage 17. The average tumor burden was 188 $\pm$ 104 mg.

Melphalan was dissolved in 0.01 *N* HCl. The drug was injected in a volume of 0.1 ml/20 g body weight, yielding a dose of 50 µmol/kg. Hyaluronidase was also applied in a volume of 0.1 ml/20 g body weight to provide an enzyme activity of 100,000 IU/kg. The enzyme preparation was dissolved in an appropriate volume of water immediately before use.

Treatment groups

Melphalan alone or both melphalan and hyaluronidase were given either i.p. or s.c. to six animals per treatment group. Melphalan was

injected i.p. into treatment groups 1–3 and s.c. into treatment groups 4–6, respectively. At 1 h prior to melphalan application, hyaluronidase was injected i.p. into groups 2 and 6 and s.c. into groups 3 and 5. Treatment group 1 served as the control for i.p. melphalan administration, whereas treatment group 4 was the control for s.c. melphalan administration. Injection of drug s.c. was carried out in the vicinity of the melanomas ca. 5 mm away from the neoplasms, with care being taken to avoid mechanical irritation of the tumor mass.

Sample acquisition and storage

The animals were killed while under i.p. ketamine/xylazine anesthesia [200 mg/kg Ketavet 100 (Parke-Davis, München, Germany) and 8 mg/kg Rompun (Bayer, Leverkusen, GER)] by cardiac puncture using heparinized syringes at exactly 1 h after melphalan administration. The blood specimens (600–1,000 µl) were immediately centrifuged at 2,000 *g* for 10 min and the plasma fractions were removed and instantly shock-frozen in liquid nitrogen. Thereafter, tissue samples were collected from the tumors localized on the right flank of the animals, from muscle (left gluteus maximus), and from the following organs: the liver (right lobe), kidney (left), and testicle. The tissue samples (50–150 mg) were instantaneously shock-frozen and stored together with the plasma samples at -78° C until analysis.

Analytical procedures

Instrumentation

Melphalan analysis was performed on a stainless-steel column (250 mm × 4 mm inside diameter) with a guard column (25 mm × 4 mm inside diameter), both of which were packed with 7-µm LiChrosorb RP-18 material (Merck, Darmstadt, Germany). The columns were mounted in a 655A-52 Hitachi column oven (Merck, Darmstadt, Germany). The HPLC system consisted of a Merck-Hitachi 655A-12 liquid chromatograph equipped with a 655A-40 auto sampler and a F1000 fluorescence spectrophotometer (Merck, Darmstadt, Germany). The chromatograms were recorded on a D-2000 chromato-integrator (Merck, Darmstadt, Germany).

HPLC conditions

The mobile phase consisted of methanol/buffer (54:46, v/v). The mobile phase buffer (MPB) contained 10 m*M* $NaH_2PO_4 \cdot H_2O$ and 2.3 m*M* SDS as the ion-pairing reagent; the pH was adjusted to 3.0 by the dropwise addition of concentrated phosphoric acid. Isocratic analyses were performed at a flow rate of 1 ml/min at 40° C. In all, 30-µl aliquots of freshly prepared solutions containing melphalan and the internal standard *N*-phenyl-2,2′-iminodiethanol (Merck, Darmstadt, Germany) were used for calibration. An excitation wavelength of 250 nm and an emission wavelength of 350 nm were used.

Sample preparation

Plasma

To 300 µl of ice-cold MPB, 200 µl of plasma and 5 µl of an internal-standard solution (4 µ*M*) were added. This mixture was passed through an activated 200-mg LiChrolut reversed-phase RP-18 cartridge (Merck, Darmstadt, Germany), where the analyte and the internal standard were adsorbed. After a wash with 2 ml of MPB, the cartridge was left at room temperature for exactly 10 min. Then the compounds of interest were eluted with 1.5 ml of methanol and collected in 2-ml polyethylene reaction vessels (Eppendorf, Hamburg, Germany). The solvent was evaporated in a vacuum concentrator (Bachofer, Reutlingen, Germany) at 40° C. The solid residue was dissolved in 200 µl of mobile phase, which was adjusted to pH 1.6 with HCl, and aliquots of 30 µl were injected for HPLC analysis.

Tissue

The frozen tissue samples (tumor, muscle, liver, kidney, and testicle) were weighed, cut up in an agate mortar with a scalpel, covered with liquid nitrogen, crumbled, and transferred to glass centrifugation tubes to guarantee efficient cooling during subsequent sonication. After the addition of 1 ml of MPB, the crude homogenates were sonicated with a type G 15 sonifier equipped with a microtip (Branson Ultraschall GmbH, Heusenstamm, Germany) at step 4 for 5 min. Sonication was applied in an ice bath, and the procedure was interrupted when the temperature of the sample rose above 5° C. The lysates were centrifuged at 3,000 g and 4° C for 10 min. Then, 200-µl aliquots of the supernatants were removed and processed as described for the plasma samples.

Statistical analysis

Significance levels of the data ($n = 6$) obtained from each treatment group were calculated with Fig. P for Windows (Biosoft, Cambridge, UK) according to the t_{HOM}- or t_{HET}-test, depending on their homogeneity or heterogeneity in the F-test [34], respectively.

Results

Characterization of the HPLC methodology

The standard curves were linear from 0.2 to 88 µ*M* for melphalan and from 0.2 to 30 µ*M* for the internal standard. The reproducibility of the assay was assessed by analysis of 74 calibration curves obtained for the 2 compounds over a period of 6 months. In the concentration range from 0.62 to 13.33 µ*M*, the relative standard deviations varied between 3.57% and 0.15% of the mean for melphalan and between 4.1% and 1.7% of that for the internal standard (concentration range 0.47–4.0 µ*M*). Melphalan and the internal standard were recovered from biological material with high precision, as became obvious from measurements using spiked FCS; a plot of the added melphalan concentration (1–10 µ*M*) versus the measured melphalan concentration yielded a straight line with a slope of 1.0003 and an intercept of -0.0405.

No endogenous substance interfered with melphalan and the internal standard, regardless of the tissue type analyzed. Thus, this technique allowed the quantitation of 40 n*M* melphalan and of 10 nmol of the internal standard/l in less than 50 mg of tissue at a signal-to-noise ratio of 10:1.

In analogy to the plasma samples, the melphalan concentrations in the various tissues were calculated on a molar basis, assuming a density of 1.0 g/cm^3 for the various tissues (the densities of plasma and the investigated tissues vary maximally by 1.8% [6]).

Effect of the route of drug administration on melphalan distribution

Melphalan (50 μmol/kg) was injected into nude mice (bearing human SK-MEL-2 melanomas) either i.p. (treatment group 1) or s.c. in the vicinity of the tumors (treatment group 4). At exactly 1 h after drug administration, samples of plasma, tumor, muscle, liver, kidney, and testicle were taken and melphalan concentrations were analyzed by HPLC. With the exception of the tumors, where local drug administration led to a 4-fold increase in the mean melphalan concentration (59 μ*M*) as compared with i.p. application of this drug (15 μ*M*), only minor effects of the route of melphalan application were observed on its distribution in the different organs (Fig. 1). As compared with plasma and muscle, where the mean melphalan concentrations were around 15 μ*M*, the drug was approx. 2-fold enriched in the liver and kidney, irrespective of the route of administration. In contrast to the tumor, plasma, and muscle, melphalan was accumulated by the testicles (ca. 2-fold) only after i.p. administration.

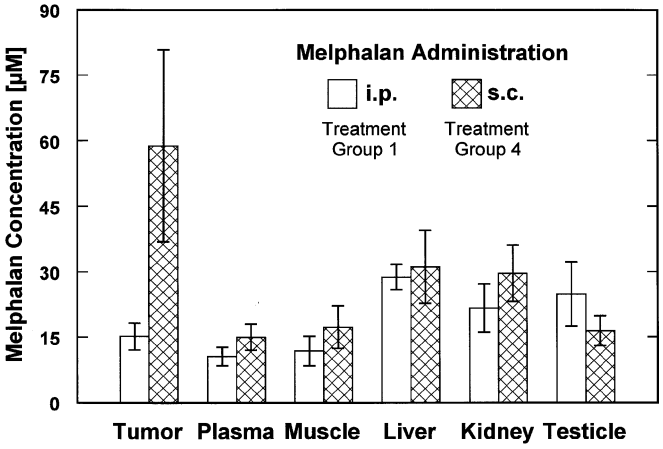

Fig. 1 Effect of the route of melphalan administration on its distribution to various tissues. In all, 50 μmol/kg of melphalan was injected into tumor-bearing nude mice either i.p. (*Treatment Group* 1) or s.c. in the vicinity of the tumors (*Treatment Group* 4). At exactly 1 h after drug administration, samples were taken and the unchanged melphalan was analyzed by HPLC ($n = 6$, mean ± SEM)

Effect of the route of hyaluronidase application on the distribution of i.p. injected melphalan to various tissues

In all, 100,000 IU kg of hyaluronidase was injected either i.p. or s.c. into nude mice bearing human SK-MEL-2 melanomas at 1 h before the i.p. application of melphalan (50 μmol/kg). At exactly 1 h after melphalan administration, samples were taken and the unchanged melphalan was analyzed by HPLC. The results of this experiment are shown in Fig. 2. As compared with the i.p. administration of melphalan alone, hyaluronidase pretreatment caused an increase in the melphalan concentration in all tissues, irrespective of the route of the administration of the enzyme. Although all but one mean melphalan concentration were evidently elevated, the accumulation of the cytostatic caused by the local pretreatment with hyaluronidase (ca. 2-fold) was significantly different from the mean tissue concentrations of the corresponding control group (i.e., after i.p. administration of melphalan alone) only in the muscle ($P < 0.002$). In addition to the well-known individual differences in pharmacokinetic measurements, the high P values (≥ 0.05) mainly resulted from shortcomings of the routine statistical procedures, which were not devised for the analysis of small sample size ($n = 6$).

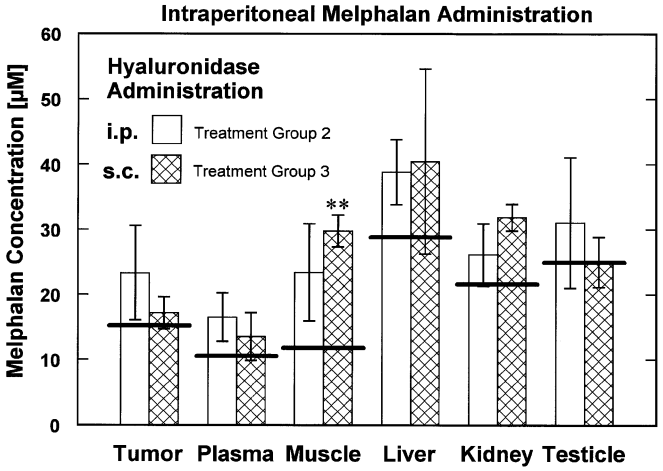

Fig. 2 Effect of the route of hyaluronidase application on the distribution of i.p. injected melphalan to various tissues. Hyaluronidase (100,000 IU/kg) was injected into nude mice (bearing human SK-MEL-2 melanomas) either i.p. (*Treatment Group* 2) or s.c. in the vicinity of the tumors (*Treatment Group* 3) at 1 h before melphalan application (50 μmol/kg). At exactly 1 h after melphalan administration, samples were taken and the unchanged melphalan was analyzed by HPLC ($n = 6$, mean ± SEM). For better comparison, the corresponding mean melphalan concentrations measured after i.p. administration of melaphalan alone (treatment group 1) are indicated by the *boldface horizontal lines* across the bars. $^{**}P < 0.002$

Effect of the route of hyaluronidase application on the distribution of s.c. injected melphalan to various tissues

In extension of the above-described experiment, 100,000 IU/kg of hyaluronidase was injected again either i.p. or s.c. at 1 h prior to melphalan administration. This time, melphalan (50 μmol/kg) was applied s.c. in the vicinity of the tumors.

As shown in Fig. 3, hyaluronidase pretreatment produced a dramatic increase in the concentration of tumor-associated unchanged melphalan as compared with s.c. administration of the chemotherapeutic alone. After i.p. injection of the enzyme a 6.6-fold rise in tumor-associated melphalan was measured ($P < 0.05$), whereas local s.c. administration caused an even higher increase by a factor of 7.8 ($P < 0.05$) as compared with the corresponding controls (treatment group 4), where melphalan alone was applied locally.

In contrast to the results obtained after i.p. administration of melphalan, in treatment groups 5 and 6 (s.c. melphalan) the extent of melphalan distribution clearly depended on the route of hyaluronidase application. With the exception of the gonads, where tissue-associated melphalan concentrations were below the respective control values (treatment group 4), s.c. hyaluronidase pretreatment obviously caused elevated melphalan concentrations in all tissues. Thus, the situation was essentially the same as that illustrated in Fig. 2.

Interestingly, the i.p. administration of hyaluronidase led to lower melphalan concentrations in plasma, muscle, liver, kidney, and testicle as compared with those measured after s.c. injection of melphalan, i.e., without the enzyme (treatment group 4). Thus, the melphalan concentrations were between 16 and 32 times higher in the tumors than in the other tissues.

Discussion

The use of hyaluronidase in cancer therapy [2, 3], its possible mechanisms of action [5, 36], and its potential application in isolated limb perfusion [36], an effective procedure in the therapy of metastasized malignant melanoma confined to the extremities [22], have been fully discussed elsewhere. Taken together, the preclinical and clinical observations suggest that hyaluronidase administration shortly before the administration of chemotherapeutics increases the access and, thus, the effectiveness of anticancer drugs in tumors [3, 5, 36]. However, this favored mechanism of action remained hypothetical due to the lack of pharmacokinetic investigations on chemotherapeutics with respect to hyaluronidase-induced accumulation by the tumor or the distribution in the body. To examine the hypothesis outlined above as well as the selectivity of such a therapeutic approach, hyaluronidase-induced distribution of melphalan was measured in tumor-bearing nude mice with respect to the mode of drug administration using reversed-phase ion-pair HPLC with fluorimetric detection of melphalan.

Despite its lability at room temperature, melphalan is known to be stable in plasma at -20°C for at least 3 weeks [9]. Therefore, the measured melphalan concentrations are supposed to represent the situation in the body at the time of sampling, i.e., at 1 h after

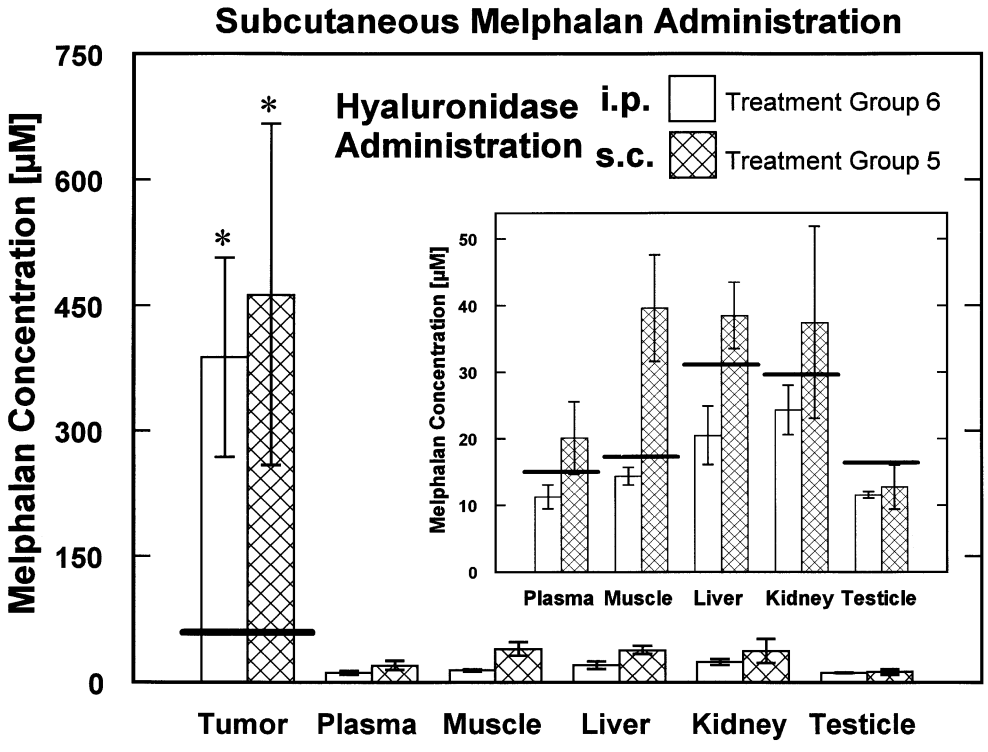

Fig. 3 Effect of the route of hyaluronidase application on the distribution of s.c. injected melphalan to various tissues. Hyaluronidase (100,000 IU/kg) was injected into nude mice (bearing human SK-MEL-2 melanomas) either i.p. (*Treatment Group* 6) or s.c. in the vicinity of the tumors (*Treatment Group* 5) at 1 h before melphalan application (50 µmol/kg). At exactly 1 h after melphalan administration, samples were taken and the unchanged melphalan was analyzed by HPLC ($n = 6$, mean $\pm$ SEM). For better comparison, the corresponding mean melphalan concentrations measured after s.c. administration of melphalan alone (treatment group 4) are indicated by the *boldface horizontal lines* across the bars. *$P < 0.05$

melphalan application, since all tissues were immediately shock-frozen in liquid nitrogen and then stored at -78°C for maximally 4 weeks. During the critical steps of sample preparation, melphalan stability was guaranteed by a combination of low temperature, relatively high chloride concentration (e.g., ca. 100 m*M* in plasma), and low pH (< 2), all factors known to retard or stop melphalan hydrolysis [7–9, 15, 16]. The solid-phase extraction procedure on 200-mg LiChrolut cartridges yielded several instances of interference with the hydrolysis products of melphalan in the different tissues, such that (*S*)-3-{4-[bis-(2-hydroxyethyl) amino]phenyl}alanine (dihydroxymelphalan) and (*S*)-3-{4-[(2-chloroethyl-2′-hydroxyethyl)amino] phenyl}alanine (monohydroxymelphalan) were not quantified.

In this study, the determination of the hydrolysis products was not essential, since dihydroxymelphalan is unreactive and the monofunctional alkylating agent monohydroxymelphalan possesses maximally 1/20 of the antitumor activity of melphalan [10]. However, it should be borne in mind that the measured melphalan concentrations refer to the unchanged drug and that melphalan and monohydroxymelphalan, which had been covalently bound to bionucleophils within the 1 h allowed for the distribution of the drug, were not extracted from the tissues.

To study hyaluronidase-induced melphalan distribution, melphalan alone or a combination of melphalan with hyaluronidase was injected either i.p. or s.c. in the vicinity of the melanomas. At 50 μmol/kg, the applied melphalan dose was comparable with the doses used in isolated limb perfusion [39]. Hyaluronidase was given at 1 h before the melphalan injection.

As shown in Fig. 1, s.c. injection of melphalan alone caused an approx. 4-fold higher melphalan concentration (59 μ*M*) in the tumors than did i.p. application (15 μ*M*). Only minor effects were observed with respect to the route of melphalan application on its distribution in other tissues. The measured melphalan concentrations were ca. 13 μ*M* in plasma, 15 μ*M* in muscle, 30 μ*M* in the liver, 26 μ*M* in the kidney, and 21 μ*M* in the testicle. In contrast to in vitro experiments on SK-MEL-24 human malignant melanoma cells, where hyaluronidase pretreatment did not augment melphalan uptake [27], the enzyme slightly increased the concentration of i.p. injected melphalan in all tissues to ca. 20 μ*M* in the tumor, 15 μ*M* in plasma, 27 μ*M* in muscle, 40 μ*M* in the liver, 29 μ*M* in the kidney, and 28 μ*M* in the testicle, irrespective of the route of hyaluronidase coadministration (cf. Fig. 2). Furthermore, s.c. injected melphalan was dramatically accumulated by the SK-MEL-2 tumors after both i.p. and s.c. administration of hyaluronidase, yielding melphalan concentrations of 388 μ*M* (i.p.) and 462 μ*M* (s.c.); (Fig. 3), corresponding to a 6.6-fold and a 7.8-fold increase, respectively, as compared with s.c. administration of melphalan alone.

This augmentation of tumor-associated melphalan gives clear evidence that systemically applied hyaluronidase can penetrate through the walls of blood vessels and that the enzyme reaches the tumors, where it exerts its hydrolytic activity. Thus, the extremely short half-life observed for hyaluronidase (several minutes) in serum in pharmacokinetical measurements [14, 21, 40] reflects not the inactivation but rather the extravasation and redistribution of the enzyme to the various tissues. The finding that melphalan is also enriched in the tumors after i.p. administration of the enzyme is an objective experimental basis for explanation of the beneficial effects described in case reports on the treatment of cancer with a combination of hyaluronidase with other cytostatics [2, 3, 32].

Although the tumor-associated melphalan concentration was somewhat lower after i.p. administration of hyaluronidase, melphalan enrichment in the tumors was more selective, i.e., the tumor-associated melphalan concentration was 16- to 32-fold higher as compared with the other tissue concentrations. In contrast to s.c. injection, i.p. administration of hyaluronidase caused a decrease in the concentration of the cytostatic in all other tissues as compared with the s.c. administration of melphalan alone (cf. Figs. 2 and 3). However, this phenomenon is not understood at present.

The extension of this work to the hyaluronidase-induced pharmacokinetics of melphalan and vinblastine, combined with tumor pharmacological and toxicological experiments using a recently established isolated limb perfusion model [26] with Rowett *rnu/rnu* rats implanted with human SK-MEL melanomas [35], will help to find the optimal dose and infusion time as well as the mode of application of a highly effective combination of chemotherapeutics with hyaluronidase (i.e., the admixture of the enzyme to the perfusate versus the infiltration of the tumor area) and, thus, provide the basis for a clinical study.

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