original paper

Potentiation of the therapeutic index of interleukin-2 immunotherapy by combination with taurine in a syngeneic murine tumour model

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Abstract

Background Administration of interleukin-2 (IL-2) is limited by the induction of the vascular leak syndrome (VLS).

Aims To examine the effect of taurine on the toxicity and antitumour activity of IL-2 in a B16 melanoma pulmonary metastases model.

Methods B16 melanoma cells were injected into female C57BL/6 mice. Macroscopic melanoma pulmonary foci were established by day 10 in untreated mice. Treated mice were randomised into treatment by rIL-2 alone, rIL-2 plus taurine or taurine alone. Control animals received saline. Mice were sacrificed on day 18. Lung metastases were counted in a blinded fashion with the aid of a dissecting microscope. Wet to dry lung weight was measured following lung removal. In another experiment animals were treated as above (n=15 per group) and survival following treatment monitored.

Result Treatment with IL-2 and taurine significantly reduced lung nodules compared with IL-2 alone. Host survival was significantly enhanced. The wet to dry (w/d) ratios of lung weights in the group receiving IL-2/taurine were significantly less than IL-2 alone. Bronchoalveolar lavage protein fluid was reduced indicating reduced pulmonary injury.

Conclusion These findings indicate that the combination of taurine with IL-2 augments the efficacy of this immunotherapy while reducing its associated dose-limiting toxicity.

Introduction

The biologic response modifier IL-2 has been shown to lead to remission of tumours in both animal models[1] and in clinical trials of melanoma and renal cell carcinoma,[2] illustrating the potential power of the immunologically-mediated mechanisms triggered by IL-2 therapy. However, its use as a single agent has been limited by a relatively low response rate against most other tumours and the severe treatment-related morbidity often observed with the doses necessary to obtain antitumour efficacy.

New strategies to enhance the antitumour activity of IL-2-based therapy are needed. Animal and laboratory studies[3-5] suggest that the antitumour effect of IL-2 could be improved using combination therapy with other cytokines or antimetastatic agents. To date, many of the agents such as dexamethasone and methotrexate which are used to reduce the severity of IL-2 therapy have had suppressive effects on cellular immune function and, in some cases, antagonised the antineoplastic effect of IL-2, resulting in no net gain in therapeutic index.[6]

Taurine, a sulphur-containing amino acid, has many physiological actions including an ability to modulate calcium homeostasis.[7,8] Through its regulation of intracellular calcium flux taurine has beneficial effects in the prevention of endothelial cell (EC) dysfunction and lysis[9] and up-regulates the antimicrobial function of human inflammatory cells.[10] It has been demonstrated both *in vitro* [11,12] and by direct histological evidence[13] that the EC injury associated with IL-2 therapy is mediated in part by activated lymphocytes. Since regulation of $[Ca^{2+}]_i$ is intimately related to lymphocyte activation[14,15] and cytolysis[16] and as lymphocytes are fundamental to the process of IL-2 immunotherapy, we hypothesized that taurine could play a valuable role in enhancing the efficacy of this therapy. This hypothesis is based on *in vitro* evidence where we have shown that taurine significantly attenuates IL-2-induced EC injury while enhancing lymphokine-activated killer (LAK) cell tumour cytotoxicity.[17]

This hypothesis was tested in an *in vivo* study using a model of metastatic melanoma. We compare the differences in toxicity, antitumour activity and survival benefit of IL-2 therapy compared with IL-2 and taurine administered to mice with established lung metastases.

Materials and methods

Animals and cells.

Female C57BL/6 mice (8-12 weeks old, ≈20 g) were purchased from Charles River UK. The animal protocols used in this study were reviewed and approved by the Department of Health and conformed to European Legislatory requirements. The murine melanoma B16 cell line was obtained from the European Collection of Animal Cell Cultures (ECACC), Wilts, UK. This cell line is syngeneic to C57BL/6 mice and was used to induce pulmonary metastases. Cells were maintained in culture medium ([CM] (DMEM supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100U/ml penicillin and

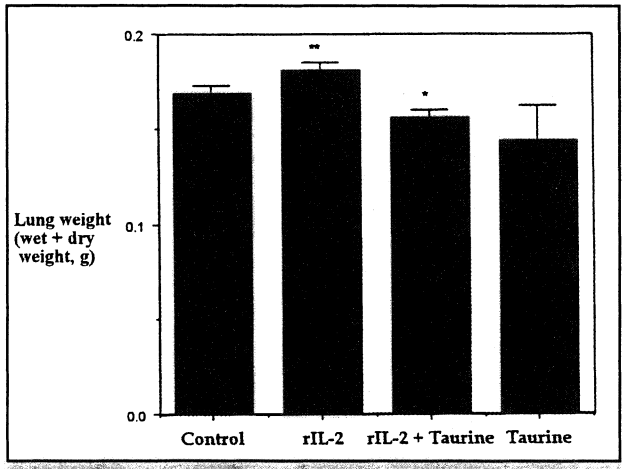

Figure 1. Lung weights as a function of treatment. C57BL/6 mice given i.p. injections as indicated. On day 18 water content of lungs assessed, lungs then lyophilized and weighed to determine dry weight. Results represent the mean±SEM of the total tissue weight; wet weight n=15/group, dry weight n=15/group. *p<0.05 versus IL-2; **p<0.01 versus control.

100g/ml streptomycin sulfate) (Gibco Life Technologists Ltd., Paisley, Scotland) at 37°C in a humidified 5% CO_2 atmosphere.

Reagents

Human recombinant IL-2 (Proleukin, Eurocetus BV, Amsterdam, The Netherlands) was kindly supplied by Chiron UK, Ltd. The human form of IL-2 is not species specific and has previously been used in the experimental immunotherapeutic manipulation of murine tumour growth.[18,19] IL-2 was reconstituted in sterile water. Taurine was obtained from Sigma Chemical Co. (Dorset, UK).

Therapeutic experiments.

The protocol for the preparation of tumour cell injections was as previously described.[1] Briefly, B16 tumour cell suspensions were prepared by trypsinization (Gibco). The cells were washed three times with Hank's balanced salt solution (HBSS [Gibco]), counted and viability tested by trypan blue dye exclusion. On day 0 mice were given injections of 3 x 10^5 cells/100ml Ca^{2+}/Mg^{2+}-free Earles balanced salt solution (EBSS [Gibco]) using the lateral tail vein, with one group receiving a sham injection of EBSS alone. This number of cells established macroscopic melanoma pulmonary foci by day 10 in untreated mice. Ten days after injection mice were randomised into treatment groups (n=15/group), including rIL-2 (50,000IU) alone, rIL-2 plus taurine (200mg/kg) or taurine alone. Control animals received saline in the same manner as treated animals.

All treatments were given as i.p. injections in 0.5ml 8 hourly for a total of 15 injections.[1] Frequent, low-dose i.p. injections of IL-2 have been shown to be more effective in prolonging serum levels and in inducing lymphocyte growth *in vivo*.[20] On day 18 after tumour injection the mice were sacrificed. Lungs were removed and metastases, which appeared as black nodules on the surface of the lung, were counted in a blinded fashion with the aid of a dissecting microscope.[21,22]

For wet to dry lung weight measurement, lungs were removed, weighed, lyophilised for 72 hours and weighed again. As an additional measurement of pulmonary damage, BAL protein content was assessed. Briefly, 1ml of saline was introduced into the lungs via a cannula and syringe. This was aspirated and followed by a further 0.5ml injection which was also recovered. Following centrifugation of the fluid (250g, 5min), 1ml of the supernatant was removed and stored at –80°C until protein measurements were carried out using Coomassie protein assay reagent (Pierce) and bovine serum albumin as a standard.

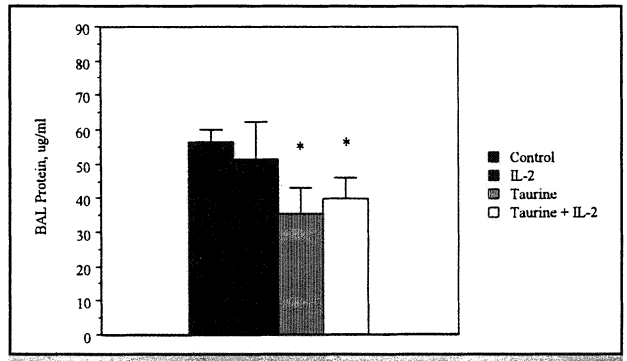

Figure 2. BAL protein levels from C57BL/6 mice bearing B16 tumours. Groups were given treatment as detailed in the materials and methods section. On day 18 animals were sacrificed, lavaged with saline and assayed for protein content by the Coomassie Blue assay. *p<0.05 versus IL-2.

In another experiment, animals were randomised to the same treatment groups as described above (n=15 per group) and survival following treatment was monitored. In compliance with the Department of Health regulations, the study period from induction of tumour did not extend beyond 60 days at which point all of the animals had expired.

Statistical analysis.

The data are presented as mean±standard error of the mean (SEM). Significance was computed by using the DataDesk (Ithaca Inc, NY) computer software program on a Macintosh Performa 5400/160 and determined by analysis of variance (ANOVA) with Scheffe post hoc correction. Statistical differences between survival curves were calculated by means of the log-rank test.[21] Probability values (p) of ≤0.05 were regarded as reaching statistical significance.

Results

Effect of taurine on vascular leakage

Taurine reduced vascular leakage induced by administration of IL-2 in the lungs of mice. The water content in the lungs of mice which had been treated with IL-2 therapy was determined as a gauge of pulmonary edema formation caused by cytokine injury. This was assessed by weighing lungs before and after lyophilisation. Total lung weights are shown in Figure 1. IL-2 therapy alone caused a significant increase in the weight of pulmonary tissue compared with saline-treated control lungs (0.181±0.004 vs 0.168±0.039g respectively), a typical manifestation of the VLS. When taurine was given along with IL-2 the weight of lungs was significantly reduced to levels comparable with the control group (0.156±0.0038 IL-2+taurine vs 0.181±0.004g IL-2). Administration of taurine alone caused a (non-significant) reduction in mean lung weight.

BAL protein levels in treated and untreated animals are shown in Figure. 2. IL-2 treatment did not result in a significant increase in BAL protein. Figure 2 shows that the lung lavage of the groups that received taurine plus IL-2 therapy contained significantly lower levels of protein than the group that was treated with IL-2 alone.

Effect of taurine and IL-2 on B16 melanoma metastases

The number of pulmonary metastases in each treatment group is summarised in Table 1. A statistically significant reduction in pulmonary metastases was seen in the group of animals receiving 50,000IU IL-2 therapy alone compared with those treated with saline (64±21 IL-2 vs 155±24 control). The group that was administered taurine alone showed a substantial (non-

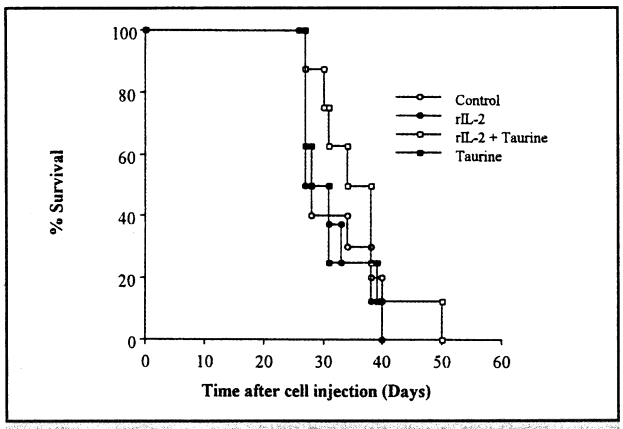

Figure 3. Effects of IL-2, taurine and combination on survival of mice injected with B16 melanoma cells. Treatment given as indicated in the methods section. n=8 pergroup. Significance levels: control versus IL-2, p=0.760; control versus IL-2+taurine, p<0.05; IL-2 versus IL-2+taurine, p<0.05.

significant) reduction in the incidence of lung nodules (111±32 taurine vs 155±24 control). Combined treatment of taurine and IL-2 resulted in an additive inhibitory effect on nodule incidence, producing a highly significant reduction in the number of nodules compared with the control group (21±29 IL-2+taurine vs 155±24 control; p<0.05). This result was also significantly less than that of animals treated with IL-2 alone.

Effect of taurine on the survival of mice given IL-2 therapy

As shown in Figure 3, administration of IL-2 with taurine prolonged host survival (p<0.05) while independent administration of IL-2 did not mediate significantly enhanced survival compared with the control group. The median survival of animals in the combination IL-2 and taurine group was 38 days compared to 28 days in the IL-2 only treatment group.

Discussion

Administration of the T cell-derived cytokine IL-2 to tumour-bearing hosts mediates tumour regression in both animal and human models suggesting a potential role in cancer therapy.[24] This form of immunotherapy, however, is associated with the development of VLS, compromising its' therapeutic potential. Thus the focus of IL-2 treatment has shifted to its use in combination with other agents in an attempt to maintain effective tumour destruction at low and well tolerated doses.[25] We combined IL-2 with taurine to examine whether it improved efficacy of IL-2 while reducing toxicity.

In this study the combination of IL-2 and taurine caused a dramatic reduction in the number of established B16 murine melanoma pulmonary metastases. Cytokine-mediated lung injury, as determined by fluid accumulation and protein lavage levels, was significantly reduced by taurine and animals treated with the combination experienced enhanced survival compared with those receiving IL-2 alone. The antitumour activity and survival compares favourably with results from other combination treatments with IL-2.[26-28]

Taurine also appears to possess antimelanoma effects. The IL-2 and taurine combination was superior to IL-2 alone. Mice treated with IL-2 alone showed a significant reduction of the number of established melanoma pulmonary metastases while IL-2+taurine treated animals showed a highly significant reduction in nodule incidence compared with both control and IL-2 groups. Although the mechanism underlying this

Table 1. Treatment of melanoma pulmonary metastases

Treatment	Dose (3 times daily)	No. of metastasesa (mean±SEM)
Control	Saline	155±24
IL-2	50,000 IU	64±21*
Taurine	200mg/kg	111±32
IL-2+taurine	50,000 IU+200mg/kg	21±29**

aC57BL/6 mice were given i.v. injections of $3x10^5$ tumor cells; therapy was initiated 10 days later and continued for 5 days.
* p<0.05 versus control, **p<0.05 versus IL-2 alone; p<0.05 versus control.

potentiation was not investigated in the present study, we have previously identified a significant role for taurine in the augmentation of lymphokine-activated killer (LAK) anti-melanoma cytotoxicity using splenic LAK isolated from animals in these same treatment groups.[28]

Administration of taurine alone reduced tumour burden, suggesting that taurine may possess an antineoplastic function, a novel property for an amino acid. It is possible that taurine either amplifies an IL-2-induced immune response or reduces tumour burden by an independent mechanism. Previous studies suggest that taurine derives its anti-tumour effects, at least in part, by increasing the cytolytic activity of IL-2-activated LAK cells.

Thus taurine may provide a stimulatory effect on IL-2 induction of LAK activity, which may partly explain the observed antimetastatic effect. We have previously shown that taurine enhances lymphocyte-mediated tumour cytotoxicity by a mechanism involving, at least in part, the Ca^{2+}-dependent enhancement of levels of cytolytic mediators in the LAK cell, possibly improving the efficacy of the 'lethal hit' of the activated cell[29]. To provide direct evidence that the *in vivo* anti-tumour effects induced by IL-2+taurine are LAK cell-dependent experiments would need to be repeated with animals depleted of these effector cells, e.g., by injection of anti-NK1.1 mAb.

This study demonstrates that injections of IL-2 and taurine decreased interstitial water accumulation in the lungs of mice. This result suggests that taurine may play a role in reducing the major dose-limiting toxicity induced by IL-2 therapy and may allow for administration of high-dose IL-2 immunotherapy with attenuation of its accompanying toxicity. The reduction in VLS is further demonstrated by the finding of decreased BAL protein which shows that vascular integrity is maintained in the combination treatment group. Again we have previously shown in vitro that the supplementation of IL-2 treated endothelial cell cultures with taurine, protects endothelial cells from IL-2 mediated cytolysis.[19]

Having demonstrated that IL-2 therapy combined with taurine is more beneficial to the host than when either agent is given alone it would be expected that co-treatment of mice with both agents would be more effective at prolonging survival. As the number of animals included in the survival study is small due to practical considerations, the conclusions that can be drawn are obviously limited. However, the results obtained showed that animals receiving combination therapy had enhanced survival compared with either a control group or with a group receiving IL-2 alone. Despite reduced tumour burden compared with the control group, the survival rates in the group administered IL-2 alone did not show improvement. This may be attributed to cytokine induced injury in this group. In contrast, the group receiving combined IL-2/taurine therapy had the benefit of both reduced lung injury and tumour

burden that resulted in prolonged survival.

The enhanced antitumour efficacy of IL-2 plus taurine therapy presented suggested that further escalation of both agents may be of therapeutic benefit. Taurine has been used in a variety of clinical situations with no known serious toxicities and has been safely administered intravenously.[30] Taurine plus IL-2 therapy may be further optimised by combination with other therapeutic agents, particularly those that further enhance the antitumour efficacy of IL-2 and that have minimally overlapping toxicities. This could form the basis for future experimental effort.

Our results demonstrate that taurine administered in vivo in combination with IL-2 can attenuate cytokine-mediated lung injury while inhibiting B16 melanoma tumour growth. The biological basis for this response, however, requires investigation and is the subject of further studies. These results indicate that IL-2/taurine combination therapy represents a novel antineoplastic therapeutic strategy and merits further investigation.

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