Cancer Immunol Immunother (1990) 32: 251–255

Cancer Immunology Immunotherapy

The antitumour activity of the interferon inducer bropirimine is partially mediated by endogenous tumour necrosis factor α

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Received 8 June 1990/Accepted 5 September 1990

Summary. Pyrimidinones, like 2-amino-5-bromo-6-phenyl-4-pyrimidinone (bropirimine), are potent immunomodulators. Natural killer cell activity and macrophage cytotoxicity are increased after bropirimine treatment, an effect exerted through induction of cytokines. Up to now, the interferons have been supposed to be the main mediators but we have found that tumour necrosis factor α (TNFα) can also be an important mediator of the bropirimine antitumour effects. Increased serum levels of TNFα were seen in rats after intraperitoneal administration of 200 mg/kg bropirimine on 2 consecutive days. We also found that the tumour-growth-inhibiting effect of the drug on a colon carcinoma in rats could be reduced about 40% by giving the rats rabbit anti-TNFα serum just prior to drug treatment. These results indicate that bropirimine can induce the release of TNFα in vivo and that this endogenous TNFα may be important as far as the antitumour effect of the drug is concerned.

Introduction

Immunotherapy is a promising modality in the treatment of cancer. Treatment with cytokines, sometimes in combination with adoptive transfer of immunocompetent cells, is successful in several animal models as well as in patients with different types of malignancies, especially renal cell carcinoma or melanoma [4, 18, 21, 28, 32]. The cytokines interleukin-2, the interferons, and tumour necrosis factor α (TNFα) have been most frequently studied. We have been working for some years with interferon γ and TNFα in various experimental tumour models [8, 14, 15, 25].

TNFα appeared to have a growth-inhibiting effect in some models [14, 15], but in those models resembling the clinical situation best, i.e. liver metastases of colon carcinoma, a significant growth inhibition could not be demonstrated despite the use of different administration schedules [25]. Therefore we, and others, concluded that TNFα should be used in combination therapy, for example with other biological response modifiers, and it has indeed been demonstrated that combining TNFα with interferon γ or α results in synergistic antitumour effects [1, 3, 27, 39].

Another suggested possibility of increasing the efficacy of exogenous TNFα, is to administer it at the time that endogenous TNFα is released. This therapy has been referred to as endogenous-exogenous therapy [9]. Examples of TNFα-inducing agents are endotoxin (lipopolysaccharide) and OK432. The former is a strong TNFα inducer but can hardly be used clinically because of severe toxicity, while the latter is a strong and far less toxic TNFα inducer [10, 26].

In search for other TNFα-inducing agents, the known interferon inducer bropirimine was tested in vivo. We examined whether it can cause TNFα release by measuring serum levels of TNFα after intraperitoneal (i.p.) administration of bropirimine in rats. Furthermore, we examined the effect of bropirimine treatment on a colon carcinoma and a rhabdomyosarcoma as compared to TNFα treatment. Finally, we examined whether the antitumour effect of bropirimine could be blocked by pretreatment with rabbit anti-TNFα serum.

Materials and methods

Animals. Male rats of the inbred WAG ($RT1^u$) strain were obtained from Harlan-CPB (Austerlitz, The Netherlands). The animals were bred under specific-pathogen-free conditions and were 10–14 weeks old when used.

Tumours. A 1,2-dimethylhydrazine-induced, moderately differentiated colon adenocarcinoma (CC531) and a rhabdomysarcoma (R2K), transplantable in syngeneic WAG rats, were used [13, 19]. CC531 was maintained in vitro in RPMI-1640 medium supplemented with 5% fetal calf serum and R2K in Dulbecco's modified Eagle medium with 10% newborn calf serum (sera were screened for virus and *Mycoplasma* infections). The media were supplemented with 1% penicillin (5000 IU/ml), 1% streptomycin (5000 μg/ml) and 1% L-glutamine (200 mM), all obtained from Gibco (Paisley, UK). Before use, the cells were trypsinized

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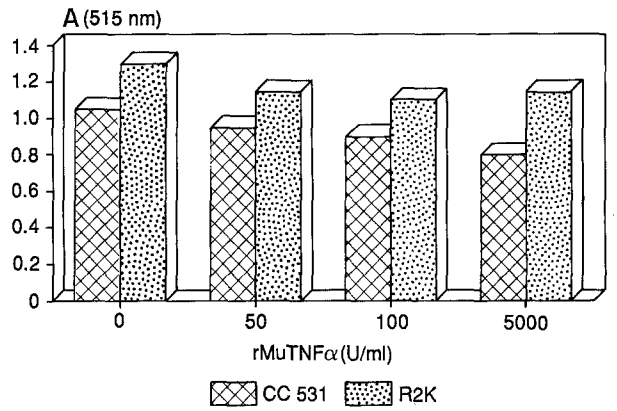

Fig. 1. Effect of different concentrations of recombinant murine tumour necrosis factor α (*rMuTNFα*)(U/ml) on in vitro growth of tumour cell lines CC531 and R2K after incubation for 48 h. Viability was measured using the MTT assay; absorbance (*A*) was measured at 515 nm

(5 min, 37° C), centrifuged (5 min, 700 *g*), resuspended in their media and counted. Viability was measured using Trypan blue (0.3% in a 0.9% NACl solution); it consistently exceeded 95%.

In vitro testing of tumour cell lines for response to TNF. Tumour cells were seeded at 1×10^4 cells/well in flat-bottomed 96-well microtiter plates (Costar, Cambridge, Mass.) in a final volume of 0.2 ml medium/well, and incubated at 37° C in 5% CO_2 for 48 h in the presence of different concentrations of recombinant murine tumour necrosis factor α (rMuTNFα). Concentrations of rMuTNFα were 0 U/ml, 50 U/ml, 100 U/ml and 5000 U/ml. Growth of tumour cells was measured using an MTT (3-4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazoliumbromide assay (for details see [25]). Absorbance was read at 515 nm using a Flow Titertek Multiskan (McLean, Va.) plate reader. Eight replicate wells were used for each concentration tested.

Agents. rMuTNFα was kindly provided by Dr. W. Fiers (Lab. Molecular Biology, Gent, Belgium). The preparation was over 99% pure, containing less than 4 ng endotoxin/mg protein. 2-Amino-5-bromo-6-phenyl-4-pyrimidinone (bropirimine) was produced as reported previously [31] and provided by the Upjohn Company, Kalamazoo, Mich., USA.

Rabbit anti-(mouse-recombinant TNFα) serum (RaTNFS) was prepared in our laboratory. Rabbits were three-times immunized with rMuTNFα in combination with Freund's adjuvants (25 μg rMuTNFα/immunization, administered intracutaneously). Blood was collected 1 week after the last immunization, serum was stored and tested for anti-TNFα activity. RaTNFS had a neutralizing capacity of 100000 U/ml serum in the L929 bioassay (see next paragraph). In vivo 0.5 ml RaTNFS given intravenously (i.v.) protected rats against the lethal effect of 20 μg rMuTNFα given subsequently. Rats not pretreated with RaTNFS ($n = 2$) both died while pretreated rats ($n = 2$) survived after rMuTNFα administration without signs of toxicity.

TNFα assay. For measurement of TNFα in the serum samples of the rats a standard cytotoxicity assay for TNFα, using the L929 cell line, was performed. Cells were seeded at 5×10^5 cells/well (100 μl) in flat-bottomed 96-well microtiter plates in the presence of actinomycin-D (final concentration 1 μg/ml). Sera, 50 and 100 times diluted, were added (to a final volume of 200 μl) and after 20 h the cell survival was estimated by the colorimetric MTT assay. Absorbance was read at 515 nm. The TNFα concentrations were calculated by comparison with a standard curve.

Experimental design. To test the sensitivity of CC531 and R2K for TNFα and bropirimine in vivo, a sub-renal-capsule assay [13, 25] was performed. In summary, a piece of tumour of about 10 mg is placed underneath the renal capsule of the rats, whereafter treatment is started. After 1 week animals are sacrificed, tumours enucleated and subsequently weighed.

Treatment consisted of i.v. injections of 4 μg/kg rMuTNFα (rMuTNFα treatment) on days 0, 2 and 4 or i.p. injections of 200 mg/kg of bropirimine (bropirimine/treatment) on days 0 and 1. Control animals received Hanks balanced salt solution (HBSS) i.v. and phosphate-buffered saline i.p. (control treatment). Each treatment was tested twice on both tumours.

In order to study the TNFα-inducing capacity of bropirimine, rats received 200 mg/kg of the drug i.p. on 2 consecutive days (the therapeutic regimen). During the second day blood samples were collected and serum was stored at –20° C. Blood samples were taken 5 min prior to the second bropirimine administration and after 1, 2, 3, 4 and 6 h. Control animals received HBSS i.p.

In another series of experiments, using the sub-renal-capsule assay model, we tested whether the antitumour effect of bropirimine against an in vivo proven TNF-sensitive tumour (CC531) could be blocked by pretreatment with 0.25 ml RaTNFS i.v. On days 0 and 1 the rats received 200 mg/kg bropirimine i.p. immediately after an i.v. injection of 0.25 HBSS, normal rabbit serum or RaTNFS.

Statistical analysis. For measurement of significance of difference in the sub-renal-capsul assay, the Wilcoxon's rank sum test was performed. For measurement of significance between control and bropirimine-treated groups in the TNFα-inducing experiments, Student's *t*-test was used for the various time points.

Table 1. Sensitivity of CC531 and R2K for rMuTNFa or bropirimine[a]

Treatment	Mean tumour weight (mg) ±SD	*n*	Tumour weight (mg)
Tumour CC531			
Control	13± 3	12	8,9,10,11,12,13,14,16,16,16,17,19
rMuTNFα[a]	7± 3	12	2,4,6,7,7,7,7,8,9,9,11,12
Control	27±11	10	10,18,22,22,24,24,26,32,35,52
Bropirimine*	10± 5	8	3,5,8,12,12,12,14,17
Tumour R2K			
Control	26±10	10	11,13,21,22,24,26,30,35,40,41
rMuTNFα	24± 9	10	11,14,17,18,26,28,29,32,33,37
Control	20± 5	12	11,14,15,17,17,19,21,22,24,25,26,28
Bropirimine*	12± 6	10	5,6,6,11,11,12,14,15,16,24

[a] In vivo sensitivity of tumours CC531 and R2K for rMuTNFα and bropirimine. A sub-renal-capsule assay was performed in rats. Control rats received i.v. 1 ml HBSS on days 0, 2 and 4 and 1 ml phosphate-buffered saline i.p. on days 0 and 1 after tumour implantation. rMuTNFα-treated rats received i.v. 4 μg rMuTNFα on days 0, 2 and 4; bropirimine-treated rats received i.p. 200 mg/kg bropirimine on days 0 and 1 after tumour implantation. Animals were sacrificed at day 7 and tumours were enucleated and weighed. Both rMuTNFα and bropirimine had a significant growth-inhibiting effect on tumour CC531 ($P < 0.01$ and $P < 0.05$ respectively). Growth of tumour R2K was significantly inhibited by bropirimine ($P < 0.01$) but rMuTNFα did not inhibit growth of R2K
* Significantly different from control

Results

In vitro sensitivity of CC531 and R2K for rMuTNFα

Both cell lines were relatively insensitive to the cytostatic/cytolytic activity of rMuTNFα as measured after 48 h incubation (Fig. 1). rMuTNFα caused only a minor, though significant, reduction in the number of viable cells in both cell lines at a concentration of 5000 U rMuTNFα/ml.

In vivo sensitivity of CC531 and R2K for rMuTNFα or bropirimine

RMuTNFα as well as bropirimine did have a significant growth-inhibiting effect on tumour CC531, while only

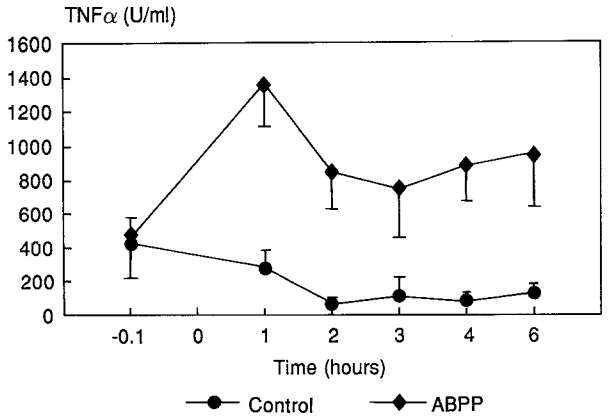

Fig. 2. Serum TNFα levels in rats after treatment with bropirimine (200 mg/kg i.p. on 2 consecutive days) or after treatment with Hanks balanced salts solution (HBSS; 1 ml i.p. on 2 consecutive days) ± SEM. Samples were taken 5 min prior to the second administration and 1, 2, 3, 4 and 6 h thereafter. TNFα was measured using the L929 bioassay. At 1, 2, 3, 4 and 6 h. TNFα serum levels were significantly increased (P <0.005 for each time point) in the bropirimine-treated group as compared to HBSS controls

bropirimine could inhibit growth of tumour R2K. Agents were tested twice on each tumour (Table 1; results of one of the experiments is shown). Efficacy for both agents was of the same order of magnitude for colon cancer CC531.

In vivo TNFα induction by bropirimine

Administration of 200 mg bropirimine/kg i.p. on 2 consecutive days (the therapeutic regimen) resulted in a significant increase in TNFα serum levels. TNFα serum levels peaked 1 h after the second administration and remained significantly elevated for up to 6 h at least. Mean TNFα serum levels in controls were surprisingly high at 5 min because two of the control rats had unexplained high TNFα starting levels.

Blocking of the antitumour effect of bropirimine against CC531 in vivo by RaTNFS

In two experiments RaTNFS partially blocked the tumour-growth-inhibiting effect of bropirimine on CC531 (Table 2). In the first experiment, the effect of bropirimine + RaTNFS was 59% of the effect of bropirimine + HBSS, while in the second experiment the effect of bropirimine + RaTNFS was 57% of the effect of bropirimine + normal rabbit serum.

Discussion

Although it has been described how bropirimine induces the release of lymphokines other than interferon [20], its TNFα-inducing capacity has never been emphasized before. This capacity could be very interesting in the light of its role in tumour therapy. So far, the antitumour effects of bropirimine were believed to be especially mediated by

Table 2. Blocking of the antitumour effect of bropirimine on colon cancer CC531 by pretreatment with rabbit anti-(mouse recombinant TNFα) serum (RaTNFS)[a]

Pretreatment/ treatment	Mean tumour growth (mg) ± SD	n	Tumour growth (mg)
Experiment 1			
Control/control	25 ± 1	12	2,13,18,25,25,25,26,26,27, 31,31,51
Control/bropirimine*	4 ± 2	10	0,1,3,4,5,5,5,6,6,6
RaTNFS/bropirimine**	13 ± 8	12	0,0,1,9,13,13,14,18,19,21, 22,22
Experiment 2			
Control/control	13 ± 5	12	5,7,7,7,10,14,14,14,17,17, 20,20
NRS/bropirimine*	3 ± 4	6	–2,–1,1,5,5,7
RaTNFS/bropirimine***	7 ± 5	8	1,2,2,4,11,11,12,12

[a] In two separate experiments the effect of pretreatment with RaTNFS on the antitumour effect of bropirimine on tumor CC531 in a sub-renal-capsule assay was measured. Animals were pretreated with HBSS (control), normal rabbit serum (NRS) or rabbit anti-TNFα serum (0.5 ml i.v. just prior to treatment), and then treated with HBSS (control, 1 ml i.p.) or bropirimine (200 mg/kg i.p.) at days 0 and 1 after tumour implantation. After 7 days animals were sacrificed, tumours were enucleated and weighed and tumour growth was calculated. In both experiments, groups treated with bropirimine, regardless of pretreatment, were significantly different from the HBSS-treated groups (P <0.01). In both experiments the groups pretreated with HBSS or NRS and subsequently treated with bropirimine were significantly different from the groups pretreated with RaTNFS and treated with bropirimine (P <0.01)

* Significantly different from control/control

** Significantly different from control/control and from control/bropirimine

*** Significantly different from control/control and from NRS/bropirimine

endogenous interferons, leading to increased natural killer (NK) cell and macrophage cytotoxicity [5, 12]. The results of this study suggest that endogenous TNFα can also be an important effector of bropirimine activity. In rats, 200 mg/kg bropirimine, given i.p. on 2 consecutive days, resulted in increased serum levels of TNFα. All rats in the bropirimine-treated group had high starting levels of serum TNFα, indicating that single bropirimine administration does also induce TNF release (TNFα serum levels were only measured after the second bropirimine administration). The high mean TNFα serum levels in the control group at 5 min were due to two of the control animals. These animals had persistently higher TNFα levels throughout the experiment than the other rats in the control group, in whose serum virtually no TNFα could be demonstrated. The tumour-growth-inhibiting effect of bropirimine against an in vivo proven TNFα sensitive tumour, could be reduced about 40% by pretreatment with rabbit anti-TNFα serum. This study shows that TNFα is only one of the mediators through which bropirimine exerts its antitumour effect, since the in vivo TNFα-insensitive tumour R2K responded as well to bropirimine as did tumour CC531. Bropirimine has already been shown to be a potent immunotherapeutic agent against different types of experimental cancer [5, 16, 17], which is confirmed by this study. Treatment with bropirimine often resulted not only in

growth inhibition but even in tumour regression. Earlier observations in our laboratory have shown that bropirimine does not only inhibit growth of tumour CC531 in a sub-renal-capsule assay but also in an artificial liver metastases model [6].

The TNFα-inducing capacity of bropirimine is the more interesting with regard to the so-called endogenous-exogenous therapy. This therapy seems to have better anti-tumour efficacy than either induction of endogenous TNFα or administration of exogenous TNFα [9]. So far, endogenous-exogenous therapy has been performed using the streptococcal preparation OK432 as the TNFα-inducing agent [26]. Like bropirimine, OK432 causes the release of TNFα [23] and it increases macrophage [23], NK cell, [33] and large granular lymphocyte cytotoxicity [29]. It also induces the release of interferon [22]. Peak levels of TNFα in serum of mice after OK432 treatment [24] were in the same order as peak levels of TNFα in serum of rats after bropirimine treatment in this study. Although comparison of results obtained in rats or mice can never lead to final conclusions, bropirimine might well be an alternative to OK432 in endogenous-exogenous therapy. It has already been reported that bropirimine and TNFα have synergistic antitumour effects [13].

This study emphasizes again that in vitro findings, as far as biological response modifiers are concerned, do not predict the in vivo outcome. Both tumours CC531 and R2K were in vitro relatively insensitive to the cytostatic/cytolytic activity of rMuTNFα, but growth of tumour CC531 was strongly inhibited in vivo by treatment with rMuTNFα.

The recognition of bropirimine as being a TNFα inducer in vivo may well explain a previously reported effect of the drug that could not be explained on the basis of its interferon-inducing capacity. It appeared that bropirimine could protect mice against listeriosis [2]. This protecting effect of the drug against an infection with *Listeria monocytogenes*, remained when the mice were pretreated with a potent anti-interferon antibody, indicating that this effect was not due to endogenous interferon. It is known that endogenous TNFα protects mice against infection with *Listeria*, especially in the early stages of the infection [7, 11], so the protecting effect of bropirimine could well be mediated by endogenous TNFα.

From the results presented in this study it can be concluded that bropirimine can induce the release of TNFα in rats; this endogenous TNFα can be important as far as the antitumour effects of bropirimine are concerned and could make the drug an even more important, immunomodulatory, antitumor agent.

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