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Relevance of tumor necrosis factor to graft-versus-host disease after small bowel transplantation

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Abstract. The small bowel (SB), an organ replete with lymphocytes, may provoke graft-versus-host disease (GVHD) after transplantation (Tx). Since tumor necrosis factor (TNF) has been suspected of mediating the tissue lesions of GVHD, we sought to determine whether TNF could be detected in the serum of rats undergoing GVHD after SBTx or lymphocyte transfer. For this purpose, postoperative serum TNF activity was determined in Lewis x Brown Norway (LBNF1) hybrid rats suffering from GVHD after undergoing transplantation of an entire (group 1; $n = 8$) or a segmental (group 2; $n = 4$) Lew SB, or after i.p. injection with lethal doses (500×10^6) of Lew lymphocytes (group 3; $n = 3$). Control LBNF1 received i.p. small doses (50×10^6) of Lew lymphocytes (group 4; $n = 4$). Serum TNF activity was assessed using the WEHI bioassay. In rats with acute and lethal GVHD after entire SBTx (group 1) or injection with large doses of lymphocytes (group 3), TNF activity gradually increased and reached high levels by the time the rats were agonal. In segmental SBTx rats (group 2), GVHD was less severe than in entire SBTx rats. Similarly, the increase in TNF activity was less intense and only transient since it had returned to control levels by the time the rats had completely recovered from GVHD. In control rats primed with small doses of lymphocytes (group 4), GVHD did not occur and no increase in TNF activity was detected. We conclude that: (1) GVHD after SBTx or lymphocyte transfer is associated with the appearance of TNF in the serum and (2) the intensity and the reversibility of this phenomenon correlate with clinical severity and lethality of GVHD. These data strongly suggest that TNF is involved in the pathogenesis of GVHD.

Key words: Small bowel transplantation, TNF, in the rat – TNF, small bowel transplantation, in the rat – GVHD, small bowel transplantation, in the rat – GVHD, TNF, small bowel transplantation

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Introduction

Tumor necrosis factor (TNF) is a cytokine that was first described as an antitumor agent [5]. TNF is now believed to be a cytotoxic monokine that plays a role in an increasing number of physiological, pathophysiological, and immunopathological events [1, 2].

There is increasing evidence that TNF plays an important role in the alloimmune response, and it has been implicated as a potential effector of allograft rejection [3, 10, 11, 15, 29] and graft-versus-host disease (GVHD) after bone marrow transplantation [18, 19, 26]. Small bowel transplantation (SBTx) can cause severe GVHD due to the large load of lymphoid tissue that accompanies the graft [17, 23, 24]. The purpose of this study was to analyze serum TNF activity in samples from small bowel transplanted rats for evidence of a possible association of TNF with GVHD produced by SBTx. In addition, we hoped to ascertain whether serum TNF activity correlated with the clinical severity and lethality of GVHD.

Materials and methods

Experimental protocol

The following experimental protocol was designed. Four groups of animals were studied. They differed according to the method used to induce GVHD and in the severity of GVHD that developed. Lewis (Lew) rats (RT1 haplotype 1/1) were used as donors and Lew x Brown Norway (LBNF1) rats (RT1 haplotype 1/n) as recipients so that GVHD alone could genetically occur [17]. The four groups included:

1. Group 1 ($n = 8$): LBNF1 were transplanted with an entire Lew SB graft.
2. Group 2 ($n = 4$): LBNF1 were transplanted with one-half of Lew jejunum.
3. Group 3 ($n = 3$): LBNF1 received i.p. lethal doses (500×10^6) of mesenteric lymph node (MLN) Lew lymphocytes.
4. Group 4 ($n = 4$): LBNF1 received i.p. minimal doses (50×10^6) of MLN Lew lymphocytes.

Table 1. Postoperative serum TNF activity in the course of GVHD following either SBTx or lymphocyte injection. A, Acute and lethal; T, transitory and nonlethal; Ab, absent

Experiments Lew to (Lew x BN)	GVHD (%)	Survival (days)	Mean percent ^{51}Cr release							
1 (n = 8) Entire SBTx	100, A	17	19	32	48	49	83	dead	–	–
2 (n = 4) Segmental SBTx	100, T	> 250	16		39		56	38	21	8
3 (n = 3) 500 × 10^6 Lip	100, A	15	8	14	57	49	dead	–	–	–
4 (n = 4) 50 × 10^6 Lip	0, Ab			11	9	7		5		5
Days post-transplantation			3	7	10	14	17	24	45	90

Microsurgical model of SBTx

The microsurgical techniques were slightly modified from those first described by Monchik and Russel [17]. In the donor, the small and large bowels were both separated by division of the right and middle colic vessels. The superior mesenteric vein was dissected free from the surrounding pancreas up to the level of the splenic vein after ligation of the pancreaticoduodenal branches. Similarly, the superior mesenteric artery was dissected free with a segment of aorta attached to it. The entire SB from Treitz's ligament to the ileocecal valve in group 1 and one-half of the jejunum in group 2 were harvested and flushed with 4 cc of cold saline solution (4 °C). In the recipient, the aorta and inferior vena cava below the left renal vein served as the receiving site for the end-to-side aortoaortic and portocaval anastomoses, respectively, using 10-0 nylon continuous suture. The proximal end to the graft was ligated whereas the distal one was drained into the recipient's ileum.

Preparation of lymphoid cells

Lew rats were killed via ether inhalation. MLN were removed, cut into fragments, and gently teased with a glass tissue grinder in modified eagle medium (MEM, Gibco Laboratories). After a brief sedimentation, the cells in the supernatant were recovered, washed twice, and resuspended in MEM at the desired cell concentration. The viable cells were counted using trypan blue dye exclusion as the criterion of viability. Adequate amounts of MLN lymphoid cells (500 × 10^6 or 50 × 10^6), in a final volume of 5 ml, were immediately injected i. p. via a small laparotomy incision into LBNF1 recipients in groups 3 and 4.

Determination of postoperative serum TNF activity

Postoperative serum TNF activity was assessed using the WEHI 164 clone 13 bioassay [7]. Briefly, ^{51}Cr release from ^{51}Cr-labeled target WEHI cells was measured in the presence of sequential dilutions of the serum samples. Recombinant rat TNF is not available so a standard curve could not be generated. Results were thus expressed in percentage of ^{51}Cr release. This methodology does not allow conclusions to be drawn regarding absolute levels of TNF reached but does allow comparisons of TNF activity between various groups and in each group at various time points. A specific sensitivity of the WEHI bioassay for rat TNF alpha was established by retesting positive samples after addition of a sheep anti-mouse TNF alpha antiserum that is crossreactive with rat TNF alpha (kindly provided by Dr. A. Meager, Division of Immunobiology, National Institute for Biological Standards and Control, Hertfordshire, UK).

Assessment of GVHD

The following indicators of GVHD were examined: (a) physical examination and observation of the typical GVHD-related signs, such as dermatitis, alopecia, diarrhea, hunched posture, weight loss, and cachexia [17, 21, 23, 24], and (b) survival rate, since recipient death is the ultimate sign of GVHD in this semiallogeneic model [17, 21, 23, 24].

Results

Postoperative course (Table 1)

LBNF1 transplanted with the entire Lew SB (group 1) developed the typical signs of acute GVHD and consistently succumbed to it within 2–3 weeks. LBNF1 transplanted with one-half of the jejunum (group 2) developed moderate signs of GVHD but finally recovered, regained weight, and survived indefinitely, as previously described [15]. LBNF1 injected i.p. with lethal doses (500 × 10^6) of Lew lymphocytes (group 3) died of acute GVHD, similar to that in the entire SBTx rats. LBNF1 injected i.p. with low doses (50 × 10^6) of Lew lymphocytes (group 4) did not show any signs of GVHD and remained perfectly healthy.

Postoperative serum TNF activity (Table 1)

In rats with acute and lethal GVHD after entire SBTx, serum TNF activity gradually increased and reached high levels by the time the rats were agonal (group 1; Fig. 1 A). In rats with less severe GVHD after segmental SBTx, the increase in serum TNF activity was less intense and only transient since it had returned to control levels by the time the rats had completely recovered from GVHD (day 90; group 2; Fig. 1 B). The increase in serum TNF activity was not specific to the method used to induce GVHD. Indeed, when lethal GVHD was produced by i.p. injection with large doses of lymphocytes, a similar increase in TNF activity could be detected as well (group 3; Fig. 1 C). Finally, in rats without any clinical evidence of GVHD, no increase in serum TNF activity could be detected (group 4; Fig. 1 D).

The WEHI bioassay, by itself, may not be able to distinguish between TNF alpha and beta. The specificity of the bioassay for rat TNF alpha, however, was established by the observation that positive sera, retested after the addition of an anti-TNF alpha antiserum, returned to baseline levels situated between 5% and 10% of ^{51}Cr release. These values were similar to the background activity measured in healthy rats in group 4 with no GVHD (Table 1).

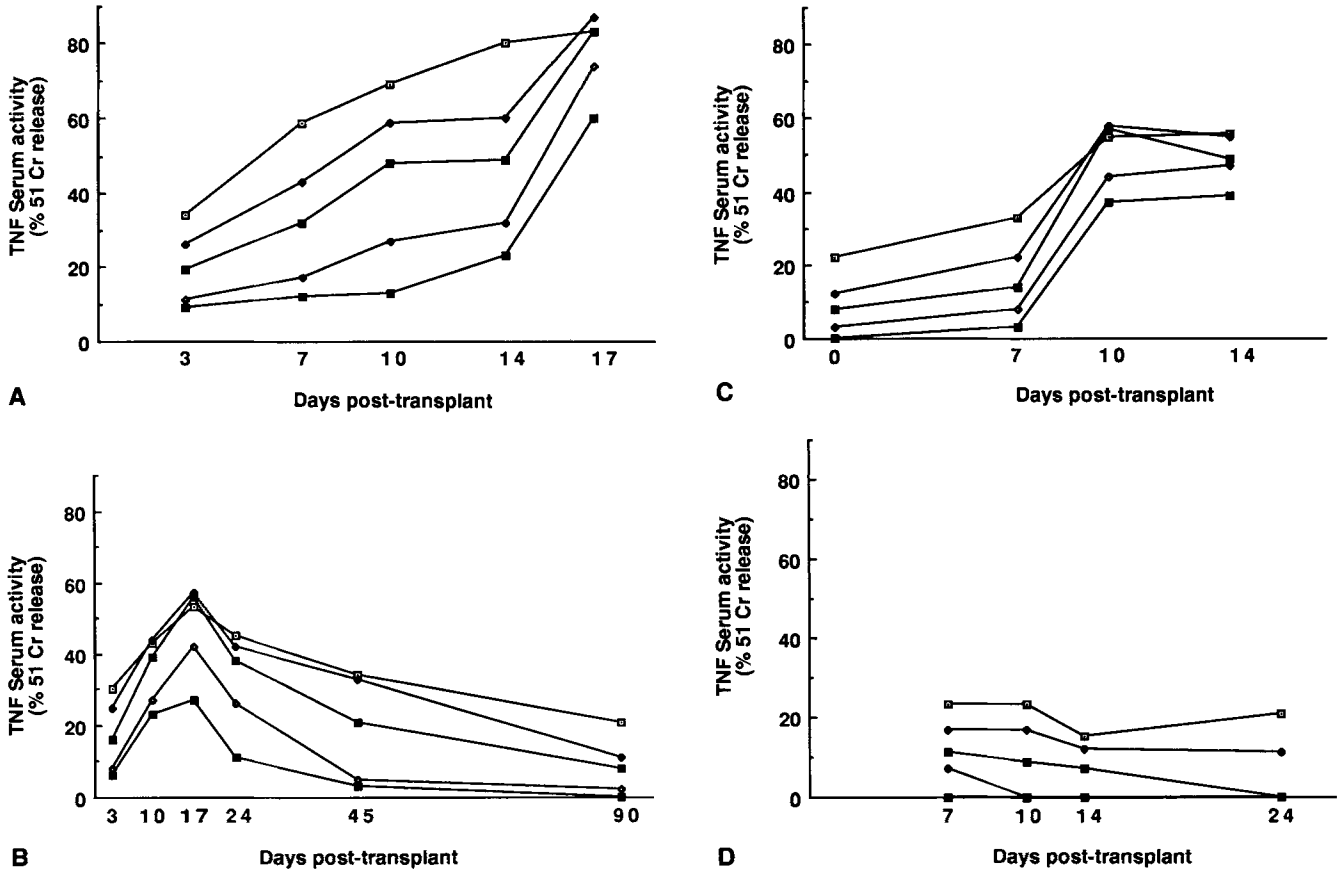

Fig. 1 A–D. TNF activity in the serum of rats: **A** with acute and lethal GVHD after entire SBTx; **B** with moderate and sublethal GVDH after segmental SBTx. –□– 1:13 dilution, –◆– 1:39 dilution, –■– 1:117 dilution, –◇– 1:351 dilution, –○– 1:1053 dilution; **C** with acute and lethal GVHD after lymphocyte injection (lethal dose 500×10^6); **D** without GVHD after lymphocyte injection (minimal dose 50×10^6). –□– 1:6 dilution, –◆– 1:18 dilution, –■– 1:54 dilution, –◇– 1:162 dilution, –○– 1:486 dilution

Discussion

Because the SB is a major lymphoid organ, it is capable of producing severe GVHD after allotransplantion [8, 17, 21, 23, 24]. Previous experiments in an animal model identical to the one used here have demonstrated that MLN lymphocytes are the main effector cells of GVHD after SBTx, whereas the gut-associated lymphoid tissue does not seem to influence this phenomenon [6, 22]. Graft-versus-host reactivity within the gut-associated lymphoid tissue, however, may be a strain-dependent phenomenon (C. L. Ingham Clark, personal communication). We have recently established that transplanted MLN provide a source of antirecipient cytolytic T lymphocytes (CTL) in the course of GVHD after SBTx [20]. In these experiments, however, no antirecipient CTL could be found within the recipient lymphoid tissues during GVHD following SBTx [20]. This strongly suggests that other mechanisms may be involved in the pathogenesis of GVHD after SBTx.

Increased levels of TNF have been detected in the course of GVHD after clinical bone marrow transplantation [28]. In another study, an increase in TNF serum levels preceded the clinical onset of acute GVHD [9]. Furthermore, two different investigators have reported that anti-TNF therapy prevents the cutaneous and intestinal lesions of the acute phase of GVHD and, thus, reduces the overall mortality due to GVHD after bone marrow transplantation [19, 26]. These experiments suggest that TNF plays a major effector role in GVHD.

Because of these associations, we sought to determine whether an association possibly existed between TNF and GVHD produced by SBTx. We made the following observations: (1) an increase in TNF activity is detected in the serum of rats undergoing GVHD after semiallogeneic SBTx; (2) an increase in TNF activity is also detected when GVHD is produced by i. p. administration of parental lymphocytes, demonstrating that the appearance of TNF in the serum reflects the graft-versus-host response rather than the method used to provoke this response (i. e., lymphocyte transfer versus solid lymphoid organ transplantation); and (3) serum TNF activity is proportional to the clinical severity and lethality of the graft-versus-host response since this activity was less important and only transient in rats with mild, sublethal GVHD and absent in rats without any clinical evidence of GVHD. Taken together, these data strongly support the hypothesis that TNF is directly involved in the pathogenesis of GVHD after SBTx.

These findings have implications with regard to the mechanisms of GVHD and the mechanisms involved in TNF production in general. Because there is a well-established association between infection and TNF [16], it is possible in our SBTx model that TNF appears in the serum as a consequence of sepsis. Indeed, sepsis is a constant feature of the terminal stage of GVHD in the SBTx model [17, 23, 24]. The native gastrointestinal tract is an important target of the graft-versus-host response, and damaged mucosal barriers are a major source of infection in the end stage of GVHD from day 12 to day 17. Endotoxins that leave the native gut and enter into the circulation could thus promote TNF production by macrophages, and this could account, at least in part, for the elevated TNF serum levels that are observed, especially in the end stage of GVHD. We believe it is unlikely, however, that sepsis alone would entirely explain the association of acute GVHD with serum TNF because increased serum levels of TNF were detected as early as day 7, at a time when there is no evidence of sepsis yet.

However that may be, the finding of an elevated TNF serum level clearly indicates that TNF is released during GVHD produced by SBTx. The cellular origin and stimuli of TNF secretion during GVHD are multifactorial. First, although T-cell lines can produce TNF, it is unlikely that donor T cells are the major source of TNF during GVHD. They are low producers compared to macrophages, and their numbers is low within GVHD target organs [18]. Second, a pathway of TNF release might involve the activation of recipient macrophages by lymphokines produced by activated donor T cells [9, 18]. Cytokines such as GM-CSF, IL-3, IL-2, and particularly interferon gamma can stimulate TNF secretion by macrophages [18]. Finally, in the end stage of GVHD, when mucosal barriers of the native gastrointestinal tract are severely injured, recipient macrophages might secondarily be stimulated by exposure to endotoxins, especially since they have already been sensitized by other T-cell-derived lymphokines such as interferon gamma and IL-2 [18]. TNF production would thus be secondarily enhanced. Further studies are needed, however, to explore in detail the exact pathways of cytokine release, both with respect to the nature of the participating cells (donor versus recipient cells) and cytokines, and to their interaction.

It can be speculated that TNF, once secreted, would affect GVHD by enhancing differentiation and function of T cells and macrophages [2], by increasing the expression of major histocompatibility complex class I and II antigens [4], by its inflammatory action, its effects on thrombogenesis, chemotaxis, and adhesion-molecule expression [14], and by a direct cytostatic effect on the target cells [27]. Interestingly, TNF has been shown to play a major role in organ allograft rejection. Elevated serum levels of TNF have been detected in the course of organ rejection [10, 15, 29], and TNF and m-RNA transcript for TNF have been found within rejected organs [12, 13]. Anti-TNF therapy has proved effective in prolonging organ allograft survival [3, 11]. Because anti-TNF treatment has been shown to prevent GVHD after bone marrow transplantation [19, 26], we hypothesize that complete inhibition of TNF might similarly ameliorate the detrimental effects of GVHD produced by SBTx. Although we did not test this hypothesis in the present experiments, a preliminary report from Scheringa et al. indicates that treatment with a TNF-neutralizing serum results in prolongation of small bowel allograft survival and abrogation of GVHD in rats [25].

In conclusion, we have demonstrated that GVHD after SBTx is associated with the appearance of TNF in the serum. The intensity and reversibility of this phenomenon correlate with both the clinical severity and the lethality of the graft-versus-host response. These results indicate a possible pathophysiological role for TNF in GVHD occuring after SBTx.

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