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Attenuation of aortic graft arteriosclerosis by systemic administration of Allotrap peptide RDP58

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Abstract There remains no treatment for chronic allograft rejection mainly manifested by progressive arteriosclerosis. We investigated the effect of Allotrap peptide RDP58 therapy on arteriosclerosis in an aortic allotransplant model. RDP58 was administered intraperitoneally at 0.1, 0.5, or 2.5 mg/kg, every other day after transplantation. RDP58 therapy markedly inhibited vascular intimal thickening, media necrosis, and adventitial cellular inflammation. The attenuation of arteriosclerosis was associated with the induction of heme oxygenase (HO)-1 expression, inhibition of TNF-α production in aortic allografts, as well as decreased specific complement-dependent cytotoxic antibodies in serum. RDP58 inhibited both smooth muscle cell (SMC) proliferation with an 80% inhibition at 100 μM without evidence of cytotoxicity and TNF-induced apoptosis of SMCs in a dose-dependent fashion. These data suggest that the suppressive effect of RDP58 on allograft arteriosclerosis is due to multiple actions of the peptide, including induction of HO-1, inhibition of TNF-α, and a direct effect on SMC proliferation.

Keywords Chronic rejection · Aortic graft · Arteriosclerosis · Smooth muscle cells · Heme oxygenase · Tumor necrosis factor

Introduction

Allograft vasculopathy, a gradually developing and progressive obliterative vascular disease in transplanted organs, is the most characteristic feature of chronic allograft rejection and the major cause of late graft loss [1, 2]. Apart from organ-specific manifestations, pathological changes of arteriosclerosis, like intimal thickening, proliferation of smooth muscle cells (SMCs), and adventitial inflammation are the most frequently observed lesions in chronic rejection [3]. Graft arteriosclerosis may be patchy and may involve large elastic arteries, small muscular arteries, or veins. The mechanism of pathogenesis of chronic rejection is still unclear. However, it is generally accepted that both immunological and non-immunological factors (metabolic stress and ischemic injury) contribute to ultimate graft loss. Based on the observation that syngeneic transplants show little or no chronic rejection, mismatch of histocompatibility is regarded as one of the major factors contributing to chronic rejection [4]. It is also well established that frequency and intensity of acute rejections correlate with the frequency of graft loss as a result of chronic rejection. These observations indicate that one or several immunological insults contribute to chronic rejection. Obviously, some of these insults cannot be prevented by conventional calcineurin-based immunosuppressive therapy [5]. For the prevention of chronic rejection, novel immunosuppressive agents that regulate both lymphocyte activation and SMC proliferation appear to be necessary.

The immunomodulatory Allotrap peptide was developed by rational design based on HLA class I-derived peptides [6]. Herein we investigated the effects of Allotrap peptide RDP58 therapy on graft arteriosclerosis in an allogeneic aorta transplant model. Systemic administration of RDP58 attenuated allograft arteriosclerosis in mouse aortic allografts. These effects were associated with induction of heme oxygenase (HO)-1 expression and inhibition of TNF-α and complement-dependent cytotoxic (CDC) antibody production. RDP58 also directly inhibited SMC proliferation and TNF-induced apoptosis in vitro.

Materials and methods

Peptides

Peptide RDP58: rnlnlnlrnlnlnlgy-CONH2 and control peptide D2RP: rvnlpialry-COOH were synthesized using Fmoc [N-(9-fluorenyl)methoxycarbonyl] chemistry, purified by HPLC and shown to be over 90% homogenous by analytical reverse phase HPLC (Synpep, Dublin, Calif.). The peptides were stored lyophilized at 4 °C and were dissolved freshly before use in a 5% mannitol in water solution. Although the original in vivo data on the immunosuppressive effect of the Allotrap peptides came from HLA class I-derived peptides, such as 1258 [7] and 2702.75-84 [8], peptide RDP58 was developed by computer-aided rational design and has no sequence similarity with 1258 and 2702.75-84.

Animals

Male inbred C57BL/10 (B10; H-2^b), C3H (H-2^k), and BALB/c (H-2^d) mice (10–12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Me.). They were maintained in a specific pathogen-free facility of the University of Pittsburgh Medical Center. The mice were provided with Purina rodent chow and tap water ad libitum. Mouse care complied with the "Principles for Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" published by NIH (No. 80-23, revised 1985).

Proliferation of SMCs

The rat vascular SMC line A10 was purchased from the American Type Culture Collection (ATCC, Rockville, Md.) and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 150 U/ml penicillin, and 150 μg/ml streptomycin at 37 °C in 5% CO_2. Cells were plated at 3×10^3/well. The A10 rat SMC line was synchronized by serum depletion and then stimulated by the addition of 10% FCS for 3 days. To determine cell proliferation, [^{3}H]TdR (1 μCi/well) was added 6 h before cell harvesting. The incorporation of [^{3}H]TdR into DNA was assessed by liquid scintillation counting. The results were expressed as mean counts per minute (cpm) ± 1SD. RDP58 was added to the cultures at various concentrations 18 h prior to the addition of [^{3}H]TdR. Peptide D2RP was used as a control.

Determination of DNA fragmentation in TNF-induced apoptosis

A10 rat SMCs (3×10^3/well) were cultured in the presence of TNF-α (400 ng/ml) for 18 h. The relative amount of apoptotic cells was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Indianapolis, Ind.) that employed antibodies recognizing histones and DNA [3]. Briefly, cells were centrifuged, washed with PBS and then re-suspended in incubation buffer. The histone-associated DNA fragments were bound by mouse anti-histone antibody, and DNA was detected with peroxidase-conjugated anti-DNA antibody. The amount of peroxidase retained in the immunocomplex was determined photometrically with 2.2′-azino-di-(3-ethylbenzthiazoline sulfonate) as a substrate. The results were expressed as enrichment factor = DNA fragmentation with peptide/DNA fragmentation without peptide.

Aortic transplantation

Allogeneic aortic transplantation was performed using B10 mice as donors and C3H mice as recipients (B10 to B10 as syngeneic control). A segment of the descending thoracic aorta approximately 10 mm in length was excised, perfused with lactated Ringer's solution, and transplanted into a heterotopic position below the renal arteries and above bifurcation in the abdomen [9]. The recipients were either treated intraperitoneally (i.p.) with RDP58 at 0.1, 0.5, or 2.5 mg/kg per day or PBS buffer (controls) every other day. Grafts were removed 10–30 days after transplantation and processed for histological examination.

Histological examinations

Aortic grafts were divided into three segments, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. The aortic graft segments were excised from the end opposite to the suture line. For evaluation of morphological changes, paraffin sections were stained with hematoxylin and eosin. Aortas from non-transplanted mice were used as normal controls. The average thickness of intima and media layers was quantified with a computer-assisted morphometry system (Quantimet 570, Leica). The number of cell nuclei in adventitia was also counted.

Immunochemistry

TNF-α or HO-1 expression in tissues was determined by an avidin-biotin-alkaline phosphatase complex (ABC) staining procedure. Cryostat sections (0.4 mm) were fixed in acetone at −20 °C for 10 min, followed by protein-blocking buffer (Shandon, Pittsburgh, Pa.). Endogenous peroxidase activity was quenched in 2% H_2O_2 before addition of rat anti-mouse TNF-α mAb (IgG1) (PharMingen, San Diego, Calif.) or polyclonal rabbit anti-rat HO-1 antibodies (Stressgen, Victoria, B.C., Canada) (both 1:200, for 1 h, at room temperature [RT]) followed by goat anti-rat or -rabbit IgG (1:400, 30 min, RT). Appropriate rat and rabbit IgG were used as isotype controls, respectively. ABC (Vector Laboratories, Burlingame, Calif.) was then added. AEC (ScyTek) was used as the substrate, and sections were counterstained with hematoxylin.

Complement-dependent cytolytic antibody activity

Serum samples from organ transplant recipients collected 10 or 30 days after transplantation were incubated at 56 °C for 30 min to inactivate complement and diluted serially in round-bottomed 96-well microtiter plates in Hank's balanced salt solution (HBSS) containing 0.1% w/v bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). After the addition of splenocytes (5×10^3) from B10 (donor, H-2^b), BALB/c (third-party, H-2^d), or C3H mice (syngeneic, H-2^k), the samples were incubated for 1 h at room temperature. The cells were then washed twice in HBSS, and 100 μl of

rabbit complement (Cedarlane, Hornby, Ontario, Canada) was added to each well. The plates were incubated for 30 min at 37 °C in 5% CO_2 in air. Subsequently, the cells were washed twice with HBSS and incubated for an additional 3 h in 100 µl HBSS supplemented with 10% FCS and 20 µl 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan (MTT, Sigma). At the end of the incubation period, the cells were washed twice, and 150 µl dimethyl sulfoxide (Sigma) was added to each well. MTT formazan was dissolved by shaking the plate vigorously, followed by centrifugation at 1430 *g* for 1 min. Optical density was measured at 550 nm using a kinetic microplate reader (Molecular Devices, Menlo Park, Calif.).

Statistical analysis

Statistical significance was assessed by Student's *t*-test or Kaplan-Meier log-rank test. A *P*-value of below 0.05 was considered statistically significant.

Results

Effect of RDP58 on proliferation of SMCs

The A10 cell line used in this study was derived from the thoracic aorta of embryonic rats and is commonly used as a substitute for primary cultured SMCs in experimental studies on SMC proliferation and death [10, 11]. To investigate the effect of RDP58 on SMC proliferation, A10 cells were first synchronized by serum depletion and then stimulated by the addition of 10% FCS for 3 days. RDP58, but not the control peptide, significantly inhibited cell proliferation in a dose-dependent manner (Fig. 1). However, the addition of RDP58 at as high as 100 µM to chromium-labeled target cells had no effect on cell lysis (data not shown), indicating that RDP58 was not toxic to A10 cells.

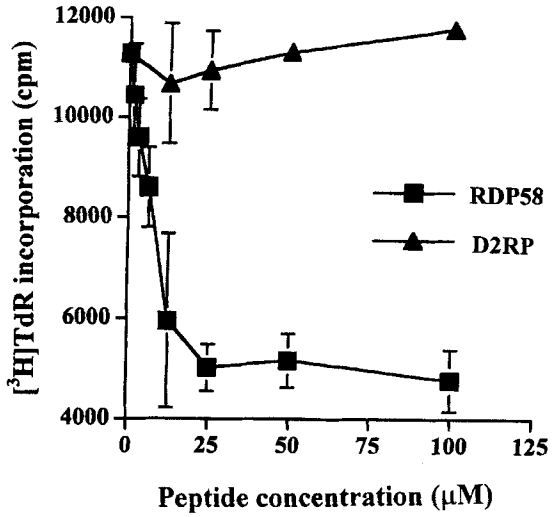


Fig. 1 The effect of RDP58 on smooth muscle cell (*SMC*) replication. The rat vascular SMC line A10 was plated in triplicate at 3×10^3/well and stimulated by the addition of 10% fetal calf serum for 3 days. [^{3}H]TdR (1 µCi/well) was added 6 h before cell harvesting. Thymidine incorporation was determined by liquid scintillation counting and expressed as mean counts per minute (cpm) ± 1SD. RDP58 or control peptide D2RP at concentrations ranging from 1.56 to 100 µM was added to the cultures 18 h prior to the addition of [^{3}H]TdR. The results are representative of three separate experiments

Effect of RDP58 on TNF-induced apoptosis in A10 rat SMCs

The induction of apoptosis in SMCs by the inflammatory cytokine TNF is an early event in the pathologic cascade of vasculopathy [12]. The addition of TNF-α to A10 cells resulted in the induction of apoptosis. Addition of RDP58 (2.5–80 µM), but not the control peptide D2RP, resulted in a dose-dependent inhibition of TNF-induced apoptosis (Fig. 2).

Effect of RDP58 on histological alternations in aortic allografts

The efficacy of RDP58 therapy on graft arteriosclerosis was tested in an aortic allograft model. Without treatment, aortic allografts from B10 mice developed significant adventitial inflammation, modest loss of medial cells, but little intimal thickening by day 10 after transplantation into C3H recipients. By day 30, aortic allografts showed advanced lesions of transplant arteriosclerosis, characterized by excessive intimal thickening, prominent loss of medial SMCs, and heavy adventitial inflammation (Fig. 3).

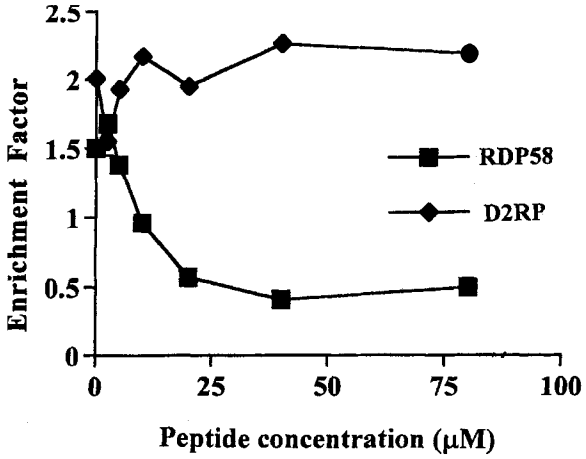


Fig. 2 The effect of RDP58 on TNF-induced smooth muscle cell (*SMC*) death. A10 rat SMCs (3×10^3/well) were cultured in triplicate in the presence of TNF-α (400 ng/ml) for 18 h. DNA fragmentation was determined by ELISA as described in the "Materials and methods" section. The results were expressed as enrichment factor = DNA fragmentation with peptide/DNA fragmentation without peptide. RDP58 or control peptide D2RP was added at the beginning of the culture. The data are representative of three separate experiments

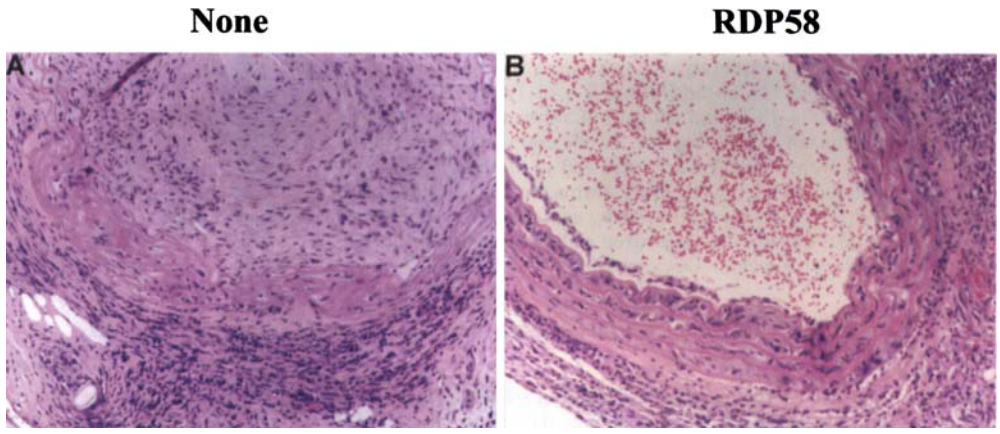


Fig. 3A, B H&E staining of B10 (H-2^b) aortic allograft sections on day 30 after transplantation (×100). **A** Aortic arteriosclerosis in non-treated recipients: intimal thickening and smooth muscle cell proliferation, loss of media nuclei, and adventitial inflammation. **B** Histological changes in aortic allografts in RDP58-treated recipients ($n = 5$ in each group). The data are representative of five experiments in each group

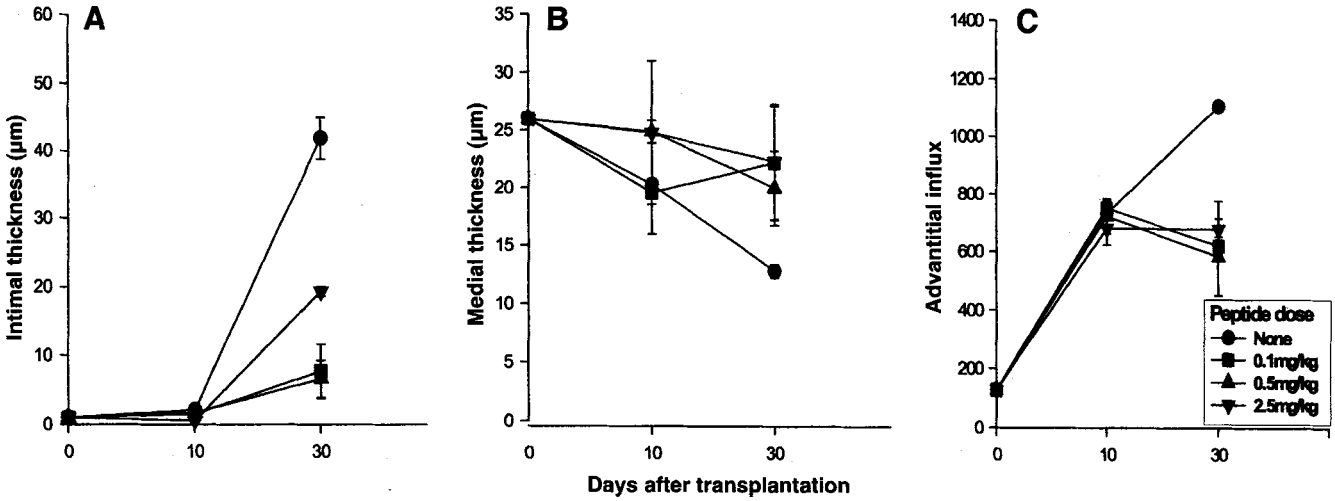


Fig. 4A–C The effect of RDP58 on histological changes in aortic allografts. **A** The cross-sectional areas of intima ($P < 0.01$, none vs any peptide group) and **B** media of aortic grafts were measured with a computer-assisted morphometry system. The results of the thickness are expressed as mean mm ± 1SD ($P < 0.05$, none vs any peptide group). **C** Number of cell nuclei in adventitia. The data are expressed as mean nuclei numbers/mm^2 ± 1SD ($P < 0.01$, none vs any peptide group). $n = 5$ in each group

Administration of RDP58 at all tested doses markedly suppressed intimal thickening examined 30 days after transplantation ($P < 0.01$ compared with untreated controls). Intimal thickening was suppressed 80% following treatment with RDP58 at 0.1 and 0.5 mg/kg. However, treatment with a higher dose (2.5 mg/kg) resulted in an only 50% reduction of intimal thickening (Fig. 4A). The reason for the loss of efficacy at higher doses is unclear but may be due to vascular toxicity of RDP58 at high concentrations.

Another manifestation of allograft arteriosclerosis, loss of SMCs in media, was also significantly reduced in all treatment groups ($P < 0.05$ compared with controls) (Fig. 4B). RDP58 therapy resulted in a modest, but significant, reduction in the number of inflammatory cells in the adventitial layer of aortic allografts ($P < 0.05$ compared with controls) (Fig. 4C).

Effect of RDP58 therapy on TNF and HO-1 expression

The expression of TNF-α and HO-1 was analyzed in frozen sections of aortic allografts and spleens from C3H recipients. TNF-α was readily detected in aortic allografts, predominantly in the adventitia, which contained a large number of infiltrating cells. RDP58 therapy (0.5 or 2.5 mg/kg i.p. every other day) significantly inhibited expression of TNF-α on both day 10 and day 30 after transplantation (Fig. 5). As expected, expression of HO-1 in the spleen of untreated and

▶

Fig. 6 HO-1 expression. Frozen sections of B10 allografts and spleen were probed with anti-HO-1 antibody on day 10 and 30 after transplantation. Expression of HO-1 in untreated and RDP58-treated recipients is shown. The results are representative of three separate experiments. The data are representative of five animals in each group

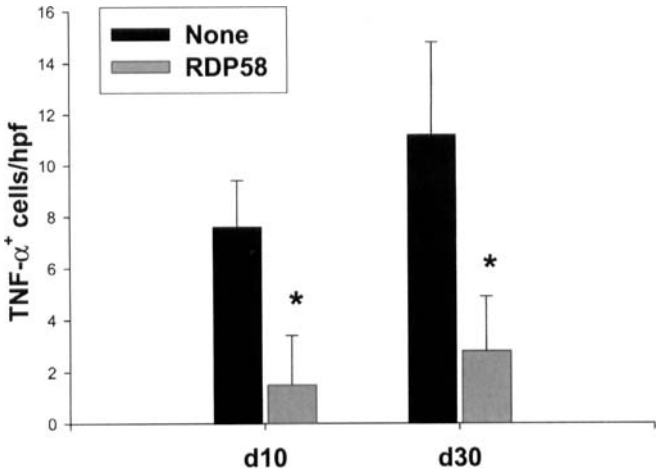


Fig. 5 Incidence of TNF-α-positive cells in adventitia of aortic allografts. Frozen sections of B10 allografts were harvested from C3H recipients that were treated with RDP58 (0.5 mg/kg per day, no treatment as controls) on day 10 and 30 after transplantation and stained with anti-TNF-α mAb as described in the "Materials and methods" section. A total of ten fields for each section was counted and the results are expressed as mean of positive cells per high power field (hpf) ± 1SD. $*P < 0.05$. $n = 5$ in each group

RDP58-treated recipients could be detected easily. In contrast, aortic allografts from untreated recipients did not display HO-1 expression. However, RDP58 therapy at doses of 0.5 and 2.5 mg/kg enhanced expression in aortic allografts and spleen on both day 10 and day 30 after transplantation (Fig. 6).

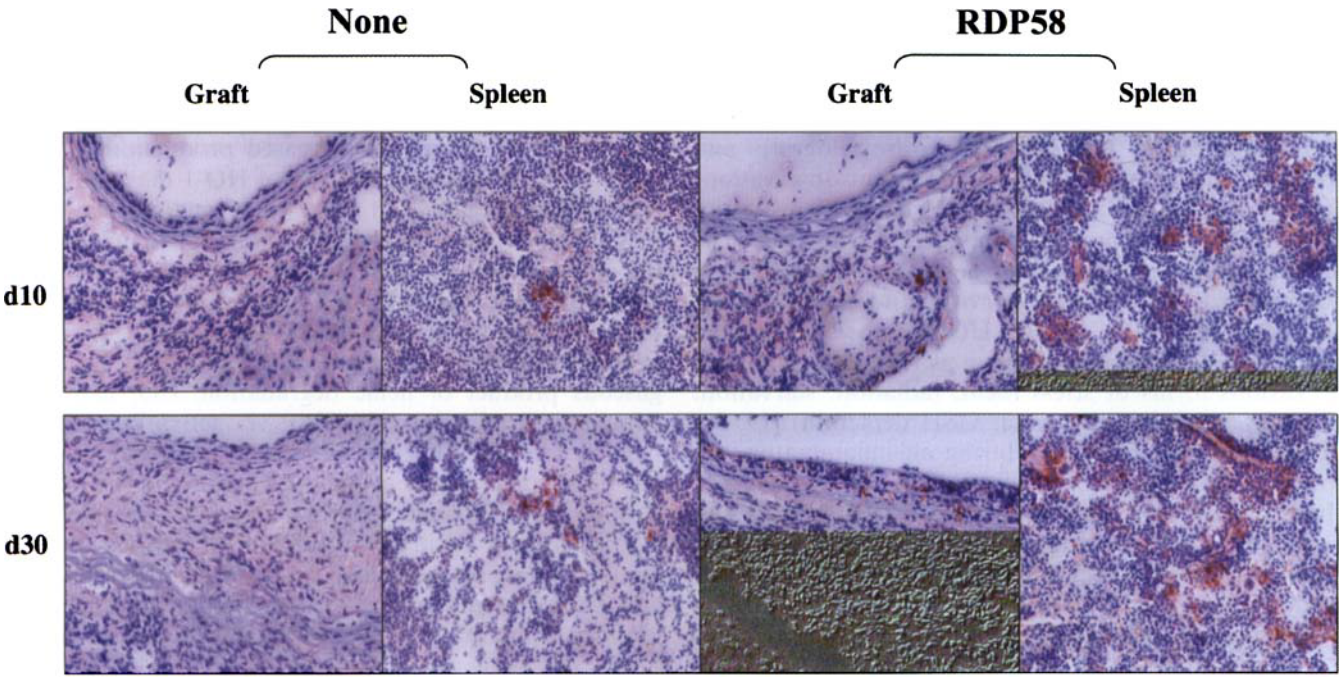
Activity of complement-dependent cytotoxicity in recipient sera

Decomplemented sera collected from C3H recipients of B10 allogeneic aorta exhibited high levels of anti-donor cytotoxicity 10 days after transplantation. Antibody levels continued to rise and reached a maximum level 30 days after transplantation (Fig. 7). No cytotoxic activity against syngeneic (C3H) or third-party (BALB/c) target cells could be detected (data not shown). Treatment with RDP58 at doses of 0.5 or 2.5 mg/kg markedly reduced the titers of anti-donor antibodies on day 10. On day 30 after transplantation the reduction of cytotoxic antibody titers in RDP58-treated recipients appeared to be less, compared with day 10 (Fig. 7). These data indicate that administration of RDP58 may not totally inhibit, but delays the production of donor-specific cytotoxic antibodies, which may partly contribute to the attenuation of the development of allograft-specific arteriosclerosis.

Discussion

Our findings demonstrate amelioration of aortic allograft vasculopathy by RDP58 therapy. Peptide treatment prevented intimal proliferation and disappearance of medial SMCs. In addition, peptide therapy reduced cellular infiltration of the aortic adventitia.

Although the underlying mechanisms associated with allograft vasculopathy are unclear, lesions developing in



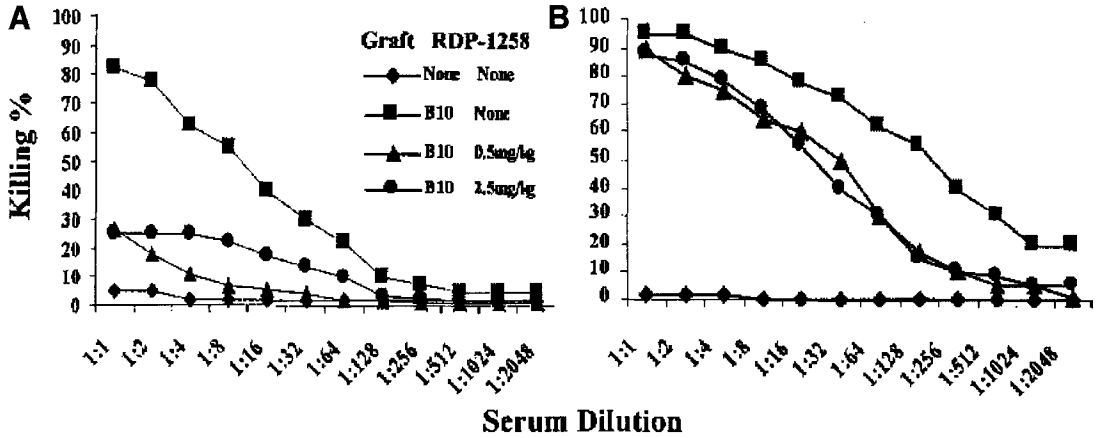


Fig. 7A, B Complement-dependent cytotoxic antibody titers. Cytotoxic anti-donor antibody titers in sera of C3H recipients treated with RDP58 (0.5 or 2.5 mg/kg per day, no treatment as control) were analyzed on (**A**) day 10 or (**B**) day 30 after transplantation of B10 aortic allograft. Sera from normal C3H mice were used as baseline controls. The results are representative of three separate experiments

allografts are thought to be initiated by immunologic and/or non-immunologic insults that result in apoptotic and/or necrotic death of SMCs in the vessel wall, followed by repair processes including SMC replication [12, 13]. Apoptotic changes in aortic allografts can be observed as early as 2 h after revascularization, and the number of apoptotic cells remains high for many weeks after transplantation. As a consequence, shortly after transplantation the number of cells in the medial layer is greatly reduced, a typical feature of allograft vasculopathy [12]. Immunologic factors contributing to the induction of apoptosis and cell death may include cytotoxic T cells, antibodies, and cytokines, in particular TNF.

The present study also demonstrates that protective effects of aortic allograft vasculopathy by RDP58 treatment were associated with the induction of HO-1 and inhibition of TNF in both aortic allografts and spleens. This is consistent with our recent observations that administration of RDP58 upregulates expression of HO-1 in other transplant models [7, 14, 15]. The induction of several protective genes has been shown to be beneficial for allograft survival and prevention of chronic rejection [16, 17, 18]. HO-1 is one such protective gene [17]. HO-1, also known as HSP 32, is induced by various forms of stress (heat, radiation, starvation, hypoxia, hyperoxia, ischemia, GSH depletion) [19, 20, 21, 22] and is upregulated during an inflammatory response and acute allograft rejection [23, 24]. Apart from stress, compounds like heme, metalloporphyrins, and heavy metals induce expression of HO-1 in vivo [20, 25, 26]. Upregulation of HO activity protects cells from oxidative injury [27] and attenuates ischemia/reperfusion injury of small bowel and liver grafts [28, 29]. Induction of HO-1 prolongs allograft survival in several rodent models [7, 14, 30]. Elevated HO-1 expression protects TNF-induced apoptosis of cultured cells [22, 30, 31]. In addition, overexpression of HO-1 appears to inhibit cell proliferation [27, 30]. It is interesting to note that at higher doses (2.5 mg/kg) the beneficial effects of RDP58 therapy were markedly lower than with lower doses (0.1 and 0.5 mg/kg). This may result from direct inhibition of HO-1 activity by higher doses of RDP5, which reverses the beneficial effects of peptide therapy [7]. More recently, the TNF-inhibitory properties of Allotrap peptide have been described [32]. The induction of protective genes like HO-1 has been shown to be associated with prolongation of graft survival [18]. Thus, expression of HO-1 inhibited graft arteriosclerosis [16, 17]. It is unclear how the upregulation of HO-1 expression mediates its protective effects. Increased HO-1 activity enables the removal and degradation of heme, a lipid-soluble, transmissible form of the potent prooxidant iron, and results in the production of biliverdin, bilirubin, and carbon monoxide [33, 34, 35]. Biliverdin has significant antioxidant and anti-complement activities [34]. Complement deposition has been observed in allografts [36]; therefore, increased production of biliverdin following overexpression of HO-1 may protect a transplanted organ from complement-mediated cell injury and attenuate chronic rejection. Bilirubin, one of the end products of heme degradation, has been shown to inhibit responses of human lymphocyte proliferation, IL-2 production, and antibody-dependent and -independent cell-mediated cytotoxicity [30, 33, 37]. The gaseous product of heme degradation, CO, like NO, stimulates production of cGMP via activation of guanylate cyclase [24, 38]. The secondary messenger, cGMP, has been implicated in cell growth arrest and the release of TNF by activated macrophages [39]. In addition, cGMP is involved in the regulation of various protein kinases, phosphodiesterases, and ion channels [39, 40, 41]. Thus, one may speculate that the increased cGMP levels may modulate several immune effector functions.

Recently, Otterbein et al. demonstrated that both in vitro and in vivo, CO at low concentrations inhibited the expression of LPS-induced TNF, IL-1β, and macrophage inflammatory protein-1β, accompanied by a simultaneous increase in the expression of LPS-induced anti-inflammatory cytokine IL-10 [42]. Besides stimulation of guanylate cyclase, CO can also modulate immune responses via inhibition of inducible NO synthase. Regulation of HO activity and NO production are intimately linked; an increased production of CO causes decreased NO production, and vice versa. Whether peptide treatment affects NO production has yet to be investigated.

Another potent effect of RDP58 is the inhibition of TNF synthesis. RDP58 has been shown to inhibit TNF in vitro in cultured monocytic cell lines as well as at the translational level in several in vivo animal models of inflammation [32]. Production of TNF during tissue injury may be responsible for the massive cell death observed in chronically rejected grafts. Tissue injury and necrosis may stimulate inflammation and thereby stimulate apoptosis, cytotoxic cellular and antibody immune effector functions. In the present study, we observed beneficial effects of low-dose RDP58 therapy (0.1 and 0.5 mg/kg) on histological integrity of the allograft. This was accompanied by decreased TNF expression and increased HO-1 levels. These observations indicate that RDP58 might exert its effects by at least two separate mechanisms to prevent chronic rejection-inhibition of inflammatory pathways and upregulation of protective pathways.

In this study, we observed a reduction of cytotoxic anti-donor antibodies in RDP58-treated recipients of aortic allografts. Presently, it is unclear if this reduction in antibody titers is a direct or indirect effect of RDP58 therapy. However, previous studies demonstrated the important contribution of cytotoxic antibodies in the development of chronic rejection [17, 43, 44]. Cardiac transplants in B cell-deficient mice developed cellular coronary endothelialitis, with destruction of the arterial media, but lack of centripetal migration of smooth muscle. These results underline the importance of antibodies in the development of chronic allograft vasculopathy, and the reduction of such antibodies may be an important factor contributing to the attenuation of chronic rejection following RDP58 therapy.

In conclusion, RDP58-mediated attenuation of arteriosclerosis in allogeneic aortic grafts may be the result of one or all of the following effects—induction of HO-1 expression resulting in protection of SMCs from apoptotic death; inhibition of proliferation of the SMC layer on activation; inhibition of TNF expression resulting in lower inflammatory injury to the graft; and lower titers of anti-donor antibodies. It is clinically significant to explore the possibility of applying HO-1 upregulators, such as RDP58, to attenuate the process of chronic rejection.

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