Ligation of HLA class I molecules on smooth muscle cells with anti-HLA antibodies induces tyrosine phosphorylation, fibroblast growth factor receptor expression and cell proliferation

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Abstract

The development of transplant atherosclerosis, a manifestation of chronic rejection, is the major obstacle to long-term survival of cardiac and renal allografts. The incidence of transplant atherosclerosis is increased in transplant recipients producing antidonor HLA antibodies following transplantation, suggesting that anti-HLA antibodies play a role in the pathogenesis of the disease. We have postulated that anti-HLA antibodies mediate the development of transplant atherosclerosis by binding to class I molecules on the endothelium and smooth muscle of the graft and transducing signals which stimulate cell proliferation. In this report we demonstrate that anti-HLA class I antibodies transduce signals in smooth muscle cells stimulating increased tyrosine phosphorylation of intracellular proteins and up-regulation of fibroblast growth factor (FGF) receptors. Antibody binding to class I molecules on smooth muscle cells is also accompanied by increased responsiveness to basic FGF and augmented cell proliferation. These findings may explain the increased occurrence of transplant atherosclerosis in recipients producing anti-donor HLA antibodies.

Introduction

Chronic rejection is the major cause of kidney, heart and liver allograft failure after the first year post-transplantation (1–3). The hallmark of chronic rejection is transplant atherosclerosis which is characterized by intimal hyperplasia. Within the areas of hyperplasia there are varying degrees of endothelial cell and smooth muscle cell proliferation as well as infiltrating macrophages (2,4). The underlying cause of chronic rejection is unknown; however, it is suspected that host alloimmune responses to disparate donor MHC antigens contribute to the pathogenesis of the disease (1–3). Anti-HLA antibodies have long been implicated in the process of chronic rejection as numerous studies have shown that patients developing antidonor HLA antibodies following transplantation are at increased risk of transplant atherosclerosis and graft loss (5–13). Intimal and myointimal deposition of Ig and immune complexes is also a common finding in transplant atherosclerosis (1–3). In addition, passive transfer of sera containing anti-donor MHC antibodies accelerates the formation of transplant atherosclerosis in experimental models of transplantation (14,15).

Studies have shown that in addition to their role in the presentation of immunogenic peptides to CD8+ T cells, MHC class I molecules are involved in signal transduction. Thus, both antibodies to monomorphic and polymorphic determinants of MHC class I have been shown to transduce signals in T lymphocytes resulting in increased intracellular Ca2+ levels (16–19), cell proliferation (17–24), IL-2 production (17,23) and IL-2 receptor expression (22). In view of these findings, we have postulated that anti-HLA antibodies may contribute to the development of chronic rejection by binding to the class I molecules on the endothelium and smooth muscle of the graft, and transducing signals which stimulate cell proliferation.

In the present study we characterized the effect of anti-
HLA antibodies on smooth muscle cells (SMC). The data demonstrate that binding of anti-HLA antibodies to MHC class I molecules expressed on SMC stimulates increased tyrosine phosphorylation of intracellular proteins, fibroblast growth factor receptor (FGFR) expression and cell proliferation. These findings indicate that anti-HLA antibodies have the capacity to transduce activation signals in SMC which may promote the development of transplant atherosclerosis.

Methods

Cells

Human aortic SMC from single donors were obtained from Clonetics (San Diego, CA), and propagated at 37°C, 5% CO2, in growth medium (EGM) containing 10 ng/ml human epidermal growth factor (EGF), 1.0 mg/ml hydrocortisone, 50 µg/ml Gentamicin, 50 µg/ml Amphotericin-B, 3 mg/ml bovine brain extract and 5% FCS (Clonetics). Assays were performed on SMC monolayers that were 70–90% confluent, and used between passages 2 and 6.

Growth factors and antibodies

Purified recombinant human basic fibroblast growth factor (rhbFGF) was obtained from R & D Systems (Minneapolis, MN). The following antibodies were used: W6/32 (IgG2b), a murine mAb that binds to a monomorphic epitope on HLA class I antigens (ATCC, Rockville, MD); mouse IgG used as a control antibody (Sigma, St Louis, MO); CD29 (IgG1), a murine mAb recognizing the integrin β1 chain (a gift from Dr E. Marcantonio, Columbia University); and goat anti-mouse IgG used for cross-linking of mouse mAb (Jackson Immunoresearch Laboratories, West Grove, PA).

Preparation of F(ab')2 and Fab fragments

F(ab')2 fragments of mAb W6/32 were prepared by digesting 10 mg of IgG with pepsin (0.1 mg/ml) in acetate buffer, pH 4.0, for 24 h at 37°C. The digested IgG was dialysed against PBS and passed over a Protein A–Sepharose CL-4B column (Sigma). The unbound fraction was collected and the purity of the F(ab')2 fragment was assessed on a 10% SDS–polyacrylamide gel. Fab fragments of mAb W6/32 were obtained by cleavage with papain. W6/32 IgG (10 mg) was digested with 0.1 mg/ml papain for 12 h at 37°C and the Fab fragment purified by Protein A–Sepharose affinity chromatography. The purity of the resulting Fab fragment was assessed on a 10% non-reducing SDS–polyacrylamide gel.

Proliferation assays

Human aortic SMC were seeded into 96-well flat bottom plates at 5000 cells/well and left to attach overnight in EGM. After 18 h of incubation, EGM was removed and replaced with EGM containing 5% FCS. On day 2, anti-MHC class I mAb or control antibodies were added to the cells and incubated for up to 48 h. Supernatants were collected from the cultures at 24 and 48 h, and the amount of bFGF was quantitated using the Quantikine Human FGF Basic Immunoassay (R & D Systems) according to the manufacturer’s specifications.

Flow cytometry

SMCs were seeded into 24-well plates at a concentration of 56,000 cells/well. After 24 h of incubation, EGM was removed and replaced with EGM containing 5% FCS. On day 3, anti-MHC class I antibodies or control mAb were added to the cells and incubated for up to 48 h. Supernatants were collected from the cultures at 24 and 48 h, and the amount of bFGF was quantitated using the Quantikine Human FGF Basic Immunoassay (R & D Systems) according to the manufacturer’s specifications.

Phosphotyrosine immunoblotting

Tyrosine phosphorylation was measured by immunoblotting SMC lysates with the anti-phosphotyrosine mAb PY20 as previously described (25). Briefly, monolayers of SMC (1 × 10^5) were incubated at 37°C for 16 h in EGM without supplements and treated with (10 µg/ml) anti-HLA mAb, isotype control mAb or EGM medium. The samples were lysed directly in SDS sample buffer, boiled for 5 min and centrifuged for 10 min at 14,000 g. The supernatant was electrophoresed on 5–20% gradient SDS-PAGE, blotted onto PVDF membrane and blocked with 5% BSA. The immunoblot was incubated with biotinylated PY20 (Zymed, San Francisco, CA) for 1 h at room temperature, washed and incubated with avidin–alkaline phosphatase (Zymed) for 1 h at room temperature. The blot was washed, developed and analyzed.

Quantitation of bFGF

SMCs were seeded into 24-well plates at a concentration of 56,000 cells/well. After 24 h of incubation, EGM was removed and replaced with EGM containing 5% FCS. On day 3, anti-MHC class I antibodies or control mAb were added to the cells and incubated for up to 48 h. Supernatants were collected from the cultures at 24 and 48 h, and the amount of bFGF was quantitated using the Quantikine Human FGF Basic Immunoassay (R & D Systems) according to the manufacturer’s specifications.

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Flow cytometry

SMCs were seeded into T75 flasks were incubated for up to 72 h in EGM media containing 10 µg/ml mAb W6/32 or 10 µg/ml isotype control IgG. Where indicated, cycloheximide (Sigma, St Louis, MO) was added at concentrations ranging from 0.01 to 0.1 µg/ml together with mAb W6/32 or isotype control antibodies. The cells were washed 3 times and detached with 0.125% trypsin/0.05% EDTA. Expression of FGFR was determined by indirect immunofluorescence on a FACScan flow cytometer as previously described (26). Briefly, SMC (0.5 × 10^5) were stained with polyclonal rabbit anti-FGFR antibodies (Upstate Biotechnology, Lake Placid, NY) for 30 min at 4°C. The cells were washed 3 times with PBS containing 2.5% FCS and 0.01% sodium azide, and stained with an FITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) for 30 min at 4°C. Cells were washed 3 times and cell fluorescence was analyzed on a FACScan flow cytometer using CellQuest Software (Becton Dickinson, Mountain View, CA). Gates for forward and side
scattering measurements were set on smooth muscle cells and a minimum of 5000 events were acquired. Instrument calibration was performed using CaliBRITE flow cytometry beads and FACScomp software (Becton Dickinson).

**Biotinylation, immunoprecipitation and Western blotting**

SMC seeded in T75 flasks were incubated for 24 h in EGM media containing 10 µg/ml mAb W6/32 or 10 µg/ml isotype control IgG. Cell surface proteins were biotinylated using sulfo-NHS-biotin (Pierce, Rockford, IL). For this, the cells were washed 3 times with ice-cold PBS (pH 8.0) and 10 ml of sulfo-NHS-LC-biotin (1.0 mg/ml in PBS) was added to the cells and incubated at room temperature for 5 min. The cells were washed 3 times in ice-cold PBS to remove unreacted biotin. The cells were lysed directly in lysis buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM iodoaceticamide, 2 µg/ml aprotinin, 1 mM PMSF prepared in TSA solution, 2 mM sodium vanadate and 10 mM sodium pyrophosphate) for 30 min on ice. The lysate was centrifuged at 14,000 r.p.m. for 10 min and the total protein content of the supernatants measured using the BCA assay system (Sigma). The amount of total protein in each supernatant was standardized and then precleared on Sepharose-4B coupled to rabbit Ig. The lysate was incubated with 10 µl of polyclonal rabbit anti-FGFR antibodies (Upstate Biotechnology) for 1 h at 4°C and immunoprecipitated with 40 µl of agarose coupled with a goat anti-rabbit IgG for 2 h at 4°C. The immunoprecipitates were washed sequentially with 1 ml of the following buffers: (i) lysis buffer, (ii) 0.1% Triton X-100, (iii) TSA solution (0.01 M Tris–HCl, pH 8.0, 150 mM sodium fluoride, 0.025% NaN3 and 2 mM EDTA) and (iv) 0.05 M Tris–HCl pH 6.8. After the fourth wash, the pellet was resuspended in 50 µl SDS sample buffer, heated for 5 min at 100°C and electrophoresed on a 6.5% SDS–polyacrylamide gel. The proteins were blotted on a PVDF membrane and blocked. The immunoblot was incubated with streptavidin–alkaline phosphatase (Zymed) for 1 h at room temperature. The blot was washed, developed and analyzed.

**Results**

**Cross-linking of class I molecules on smooth muscle cells stimulates cell proliferation**

Quiescent human aortic SMC were exposed to the anti-class I mAb W6/32 for 24–72 h and cell proliferation was measured by thymidine incorporation. Incubation of SMC with medium containing mAb W6/32 led to a 4-fold increase in the amount of [3H]thymidine incorporation at 24, 48 and 72 h (Fig. 1). In parallel control cultures, endothelial cell growth factors had a similar effect on cell growth, inducing a 6-fold increase in cell proliferation at 24 h, and 5-fold increases in cell proliferation at 48 and 72 h. In contrast, the addition of isotype control antibodies, or antibodies to SMC surface antigens other than HLA, such as CD29, had no effect on cell proliferation. Stimulation of cell growth was observed at concentrations of anti-MHC antibodies ranging from 1.0 to 20 µg/ml with maximal cell proliferation at a concentration of 10 µg/ml.

The capacity of anti-HLA antibodies to stimulate the proliferation of SMC was confirmed in experiments in which cell proliferation was measured by direct cell counting. The addition of mAb W6/32 to SMC resulted in a doubling of the cell number at 24, 48 and 72 h of study (Fig. 1). In contrast, murine isotype control antibodies did not stimulate cell growth. These results indicate that ligation of class I molecules by anti-HLA antibodies induces SMC proliferation.

To determine if cross-linking of HLA class I molecules is required to stimulate SMC proliferation, SMC were treated with a Fab fragment of mAb W6/32, in the presence or absence of a cross-linking antibody. As shown in Fig. 2, SMC reacted with the W6/32 Fab alone failed to proliferate, whereas SMC treated with W6/32 Fab followed by cross-linking with goat anti-mouse IgG proliferated strongly. To exclude the possibility that signaling was occurring via a Fc receptor SMC were treated with F(ab’)2 fragments of mAb W6/32. The addition of F(ab’)2 fragments of W6/32 to cultures of SMC resulted in a proliferative response similar to that observed when intact W6/32 IgG was used, indicating that signaling was mediated via the MHC class I molecule and not through Fc receptors. These experiments demonstrate that cross-linking of HLA class I molecules is required to deliver the signals that induce SMC proliferation.

**Effect of anti-HLA class I antibodies on FGFR expression by smooth muscle cells**

To determine whether anti-HLA antibodies stimulate cell proliferation by regulating responsiveness to FGF, we evaluated the ability of W6/32 to stimulate SMC growth in the presence of neutralizing antibodies to bFGF. The addition of anti-bFGF neutralizing antibodies significantly inhibited W6/32-induced cell proliferation in a dose-dependent manner over the 72 h

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**Fig. 1.** Stimulation of smooth muscle cell proliferation by anti-class I mAb. Quiescent SMC were cultured with 10 µg/ml of anti-class I mAb W6/32, 10 µg/ml mAb to CD29, 10 µg/ml murine isotype control IgG or EGM. [3H]Thymidine incorporation was determined when the mAb were added, and after 24, 48 and 72 h. The data are expressed as the mean c.p.m. of triplicate determinations (SD < 10%). One of eight representative experiments is shown. Cell counting was performed by Trypan blue exclusion at the time mAb were added, and after 24, 48 and 72 h. The data are expressed as the mean count of triplicate determinations (SD < 10%). One of three representative experiments is shown.
HLA antibodies stimulate smooth muscle cell proliferation

Fig. 2. Cross-linking class I MHC molecules on SMC stimulates cell proliferation. Quiescent SMC were cultured with 10 µg/ml of anti-class I mAb W6/32, 10 µg/ml mAb W6/32 Fab, 10 µg/ml W6/32 Fab followed by cross-linking with 10 µg/ml goat anti-mouse Ig, 10 µg/ml W6/32 F(ab')2, or 10 µg/ml murine isotype control IgG followed by cross-linking with 10 µg/ml goat anti-mouse IgG. [3H]Thymidine incorporation was determined when the mAb were added, and after 24, 48 and 72 h. The data are expressed as the mean c.p.m. of triplicate determinations (SD < 10%). One of four representative experiments is shown.

Fig. 3. Inhibition of W6/32-induced SMC proliferation by anti-bFGF neutralizing antibodies. SMC were treated with 10 µg/ml mAb W6/32 in the presence and absence of neutralizing antibodies to bFGF (5–0.01 µg/ml), PDGF (5–0.1 µg/ml) or TGF-β (5–0.1 µg/ml). In control experiments, SMC were incubated in EGM in the presence and absence of neutralizing antibodies to bFGF (5–0.01 µg/ml). [3H]Thymidine incorporation was determined after 24, 48 and 72 h. The data are expressed as the mean c.p.m. of triplicate determinations (SD < 10%). The results of cell proliferation at 24 h are presented. One of five representative experiments is shown.

period studied (Fig. 3). In contrast, neutralizing antibodies to other growth factors produced by SMC, such as TGF-β and PDGF showed no inhibition of W6/32-induced SMC proliferation. The specificity of the anti-bFGF neutralizing antibodies was confirmed in experiments which showed that the anti-bFGF neutralizing antibodies specifically inhibited SMC proliferative responses to bFGF but not to other growth factors such as EGF and PDGF (Table 1). These studies suggested that ligation of class I molecules by anti-HLA antibodies triggers SMC proliferation by increasing bFGF synthesis and/or bFGF uptake.

To determine if class I ligation leads to an increased production of bFGF, SMC were deprived of growth factors for 24 h, stimulated with mAb W6/32 and the amount of bFGF present in the culture supernatant was measured after 48 h. There was no difference in the amount of bFGF present in supernatants from SMC incubated with mAb W6/32 when compared to supernatants from SMC treated with mIgG control antibodies (data not shown). We next determined whether ligation of class I molecules on SMC alters FGFR expression. SMC monolayers were treated with mAb W6/32 for 24 h and the percent of FGFR-positive cells was determined by FACS analysis (Fig. 4). In the presence of mAb W6/32,

Table 1. Anti-bFGF neutralizing antibodies specifically block SMC proliferative responses to bFGF

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Growth factor</th>
<th>[3H]Thymidine incorporation</th>
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<tbody>
<tr>
<td>Control IgG (10 µg/ml)</td>
<td>52,684a</td>
<td>50,616</td>
</tr>
<tr>
<td>Anti-bFGF (10 µg/ml)</td>
<td>25,015</td>
<td>52,961</td>
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Table 4a

a[3H]Thymidine incorporation was determined 24 h following the addition of growth factor and neutralizing antibodies. The data are expressed as the mean triplicate determinations (SD < 10%).
HLA antibodies stimulate smooth muscle cell proliferation

Fig. 5. Immunoprecipitation of FGFR on SMC treated with anti-HLA antibodies. SMC were treated with isotype control IgG (A) or the anti-class I mAb W6/32 (B) for 24 h and the cell surface proteins were biotinylated with sulfo-NHS-biotin. The cells were lysed and immunoprecipitated with an antibody specific for FGFR. After SDS-PAGE, biotinylated FGFR were visualized by Western blot analysis using streptavidin–alkaline phosphatase. One of two representative experiments is presented.

Table 2. Time course of FGFR expression on SMC stimulated with anti-HLA class I antibodies

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cyclohexamide (µg/ml)</th>
<th>FGFR-positive SMC (%)</th>
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<tr>
<td></td>
<td>1 h&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 h</td>
</tr>
<tr>
<td>mIgG</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>W6/32</td>
<td>0.1</td>
<td>56</td>
</tr>
</tbody>
</table>

<sup>a</sup>Time (h) of exposure of SMC to mAb. Data are expressed as percent positive cells. A total of 10,000 events were counted per sample. One of four representative experiments is shown.

54% of the cells expressed FGFRs whereas in the absence of mAb W6/32, <2% of cells were FGFR-positive. The capacity of anti-HLA antibodies to augment FGFR expression was further substantiated in experiments in which the amount of cell surface FGFR was measured by immunoprecipitation and Western blotting. SMC were treated with the anti-class I mAb W6/32 for 24 h and the cell surface proteins were biotinylated with sulfo-NHS-biotin. FGFR were subsequently immunoprecipitated with anti-FGFR polyclonal antibodies, immunoblotted and the FGFR biotinylated proteins detected with streptavidin–alkaline phosphatase. As shown in Fig. 5, there was an increase in the amount of FGFR protein immunoprecipitated from SMC treated with anti-HLA class I mAb (Fig. 5B) compared to SMC treated with isotype control antibody (Fig. 5A).

To investigate the time course of FGFR expression following class I ligation, SMC were treated with mAb W6/32 for various time intervals and tested for FGFR expression (Table 2). FGFR expression was up-regulated as early as 1 h after exposure of SMC to anti-MHC mAb; expression peaked by 24 h and stayed at high levels thereafter. These results indicate that anti-HLA antibody binding to class I molecules on SMC transduces signals that lead to an increased expression of FGFR.

To determine if FGFR up-regulation is dependent on protein synthesis, SMC were exposed to mAb W6/32 in the presence of cyclohexamide (Table 2). Treatment with cyclohexamide did not inhibit W6/32-induced FGFR expression at 1 h. In contrast, a marked reduction in FGFR expression was observed at 6 h. These results suggest that early W6/32 induced FGFR expression does not require protein synthesis. However, in order to sustain FGFR expression following class I ligation, protein synthesis is necessary.

To determine if increased FGFR expression is accompanied by increased responsiveness to bFGF, quiescent SMC were stimulated with W6/32 in the presence and absence of rhbFGF, and cell proliferation was measured by [³H]thymidine incorporation (Fig. 6). The addition of rhbFGF to W6/32-treated SMC resulted in a proliferative response that was ~2 times greater than cultures treated with W6/32 alone and 2.5 times greater than SMC cultures treated with bFGF alone. These results show that following class I ligation, SMC proliferative responses to bFGF are enhanced.

Anti-HLA class I antibodies stimulate tyrosine phosphorylation in SMC

The ability of mAb to class I HLA antigens to induce tyrosine phosphorylation of intracellular proteins in SMC was examined. SMC were treated with the anti-class I mAb W6/32 for various periods of time and cell lysates were electrophoresed and immunoblotted with the anti-phosphotyrosine mAb PY20. The results of one of five representative experiments are
HLA antibodies stimulate smooth muscle cell proliferation

Fig. 7. Tyrosine phosphorylation studies of SMC following incubation with anti-MHC class I antibodies: (A) SMC treated with 10 µg/ml mouse IgG for 3 min, (B) SMC treated with 10 µg/ml W6/32 IgG for 3 min and (C) SMC treated with EGM for 3 min. The result of one of five representative experiments is presented.

shown in Fig. 7. Treatment of SMC with W6/32 for 3 min resulted in an increase in tyrosine phosphorylation of cellular proteins at approximate molecular masses of 60, 45, 40 and 27 kDa (Fig. 7B). Increased tyrosine phosphorylation was not observed when SMC were treated with an isotype control antibody (Fig. 7A). Treatment of SMC with medium containing EGF resulted in a similar pattern of tyrosine phosphorylation as found in W6/32-treated cells with the exception of an additional phosphorylated protein at an approximate molecular mass of 170 kDa (Fig. 7C). The phosphorylated 170 kDa protein is most likely the EGF receptor, which becomes phosphorylated following binding of EGF contained in the culture medium. These results demonstrate that the binding of anti-HLA antibodies to HLA antigens expressed by SMC transduces signals resulting in increased protein tyrosine phosphorylation.

Discussion

A form of accelerated atherosclerosis, termed transplant atherosclerosis, is the leading cause of late heart and kidney graft loss, and is estimated to affect >40% of recipients within the first 5 years following transplantation (1–3). The histologic appearance of transplant atherosclerosis shows marked proliferation and hyperplasia of vascular smooth muscle cells and endothelial cells. These findings suggest that augmented SMC responsiveness to growth factors contributes to the pathogenesis of the disease. Indeed, recent studies have identified increased expression of several SMC and endothelial cell growth factors and their receptors in areas of intimal hyperplasia. Focal increases in PDGF and its receptor have been found in the vessel wall of renal and cardiac allografts undergoing chronic rejection (27,28). Furthermore, both acidic FGF and its receptors are increased following cardiac transplantation (28,29). The expression of bFGF and FGFR1 isoforms has also been shown to be up-regulated in the vessels of cardiac and renal allografts undergoing chronic rejection (30,31).

The etiology of transplant atherosclerosis is poorly understood, yet it has long been suspected that anti-HLA antibodies are involved in the development of vascular lesions. In this report, we demonstrate that anti-HLA antibodies can contribute to the development of transplant atherosclerosis by binding to MHC class I molecules on SMC and transducing signals which lead to cell proliferation.

Our studies show that FGFR expression is rapidly induced on the surface of SMC following ligation of class I molecules by anti-MHC antibodies. The FGFR family consists of at least four distinct gene products (FGFR1–FGFR4) each composed of an extracellular ligand-binding domain that contains three Ig-like domains, a single transmembrane domain and a cytoplasmic domain that contains protein tyrosine kinase activity (32). FGF induce cell proliferation by binding to FGFR receptors and stimulating receptor autophosphorylation (33). FGF binding further triggers a series of downstream events, including activation of p21ras and mitogen-activated protein kinases, and activation of nuclear transcription factors culminating in cell proliferation (34–38). This suggests that anti-HLA antibody induced expression of FGFR1 with tyrosine kinase activity renders the SMC responsive to FGF and stimulates cell proliferation. Consistent with this interpretation, we found that induction of FGFR expression by anti-HLA antibodies was accompanied by increased SMC responsiveness to bFGF and augmented cell proliferation. Our data are also consistent with the effect of anti-MHC class I antibodies on resting T cells which proliferate following exposure to anti-class I antibodies (16–23). However, in the case of T lymphocytes, engagement of class I MHC molecules provides a signal inducing IL-2 receptor expression (22). Thus, class I signaling events result in different functional outcomes depending on the cell type investigated.

The results obtained with cyclohexamide, a protein synthesis inhibitor, indicated that early, class I-mediated induction of FGFR did not require de novo protein synthesis. This suggests that signal transduction via class I MHC leads to the rapid release of intracellular stores of FGFR. However, since FGFR expression declined after treatment of SMC with W6/32 and cyclohexamide for 6 h, sustained expression of FGFR appears to require newly synthesized FGFR.

Cross-linking class I molecules by reacting them with bivalent IgG molecules was required for the generation and transduction of an activation signal, since monovalent Fab fragments were unable to transduce proliferative signals. A
similar requirement for cross-linking has been described for class I signaling in T lymphocytes (17,18,39). Previous studies have also shown that the intensity of signal transduction is related to the number of class I molecules aggregated on the cell surface (39). In accordance with this report, we observed a higher rate of SMC proliferation when W6/32 was added at concentrations between 5 and 10 µg/ml. Only minimal proliferation was observed when anti-MHC antibodies were added to SMC at concentrations <1 µg/ml.

Antibody cross-linking of MHC class I molecules stimulated tyrosine phosphorylation of SMC proteins at a approximate molecular masses of 60, 45, 40 and 27 kDa. Elimination of the nature of the SMC proteins which become phosphorylated following class I ligation requires further studies; however, it is tempting to speculate that the 45 kDa protein is the class I molecule itself, since the cytoplasmic domain of the class I heavy chain contains serine, threonine and tyrosine residues that can be phosphorylated (40,41). It is unlikely, however, that class I molecules interact directly with cytoplasmic tyrosine kinases to transduce signals since recent studies by Gur et al. have shown that most of the intracellular portion of MHC class I molecule is not needed for signal transduction (42). Thus, class I molecules most likely associate with other molecules that have the capacity to transduce signals or to generate intracellular messengers. It has been demonstrated that class I MHC molecules can associate with a variety of cell surface molecules including hormone receptors such as insulin receptor and EGF receptor (43–46). It is possible that one or more of these molecules are involved in class I signaling. A similar requirement for accessory signal transducing molecules has been described for signaling via class I molecules in T lymphocytes. Thus, it was observed that transmission of activation signals to T cells via class I MHC molecules requires CD3 expression (23,39,47).

We have shown that signal transduction events occurring after ligation of class I molecules on SMC induces FGFR expression, but the precise mechanism by which this occurs is not yet to be determined. In previous studies we demonstrated that anti-HLA class I antibodies were capable of transducing signals in endothelial cells, stimulating increased tyrosine phosphorylation and inositol phosphate generation (25). Together, the previous results and our current findings are consistent with a model in which increased permeability of the endothelium of the graft during immune and/or inflammatory responses results in unregulated transit of cells and solutes, including anti-HLA antibodies, from the blood to the subendothelium. Binding of anti-HLA antibodies to class I molecules on the surface of SMC causes stimulation of protein tyrosine kinases, tyrosine phosphorylation and the activation of phospholipase C. These events result in the hydrolysis of phosphatidylinositol and the generation of inositol-1,3,4-triphosphates and Ca^{2+} flux. Since this same pathway is utilized by a variety of hormones, neurotransmitters as well as growth factors, it is likely that antibody ligation of class I molecules stimulates protein phosphorylation events and second messengers which also regulate FGFR expression. For example, phospholipase C is activated following class I signaling (47) and is a known substrate of the FGFR (33).

In conclusion, our data indicate that chronic rejection can be mediated by anti-HLA antibodies which bind to the smooth muscle cells of the graft and transduce signals which stimulate FGFR expression and augmented cell proliferation. Thus, prevention of transplant atherosclerosis will require interruption of the autocrine and paracrine effects associated with this growth factor receptor (48). Alternatively, since T_{h} cells produce the lymphokines required for growth and maturation of alloantibody producing B cells, and are therefore primary regulators of anti-HLA antibody production, therapy aimed at specific ablation of such cells by use of TCR antagonists and vaccines may also prove to be an effective therapeutic intervention.

The current findings may also have broader implications for the role of class I signaling in non-transplant-associated atherosclerosis. Although atherosclerosis and accelerated transplant-related atherosclerosis differ in their speed of onset and angiographic appearances, there are several parallels between these two processes. The intimal lesions in both diseases contain proliferating smooth muscle cells (2,4,49), both processes have been related to humoral and cellular immune responses (1–3,50), and both have been associated with increased levels of FGF and FGFR expression (28–31,51). It is interesting to speculate that ligands such as anti-MHC autoantibodies (52), auto-reactive T cells (50) and viral or bacterial products (53) which recognize class I molecules may play a role in the development of non-transplant-associated atherosclerosis by stimulating SMC proliferation.

Acknowledgements

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Abbreviations

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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>EGM</td>
<td>endothelial cell growth medium</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>rhbFGF</td>
<td>recombinant human basic fibroblast growth factor</td>
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<td>SMC</td>
<td>smooth muscle cells</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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