J Korean Med Sci 2001; 16: 397-406
ISSN 1011-8934

Expression of NF-κB and Cytokines in Chronic Rejection of Transplanted Murine Heart

The heart transplantation-associated accelerated graft arteriosclerosis (AGAS) is one of the major causes of cardiac allograft failure. We investigated the early time-course of expresssion patterns of cytokines, transcription factor, and its inhibitor in the intraabdominally transplanted mice hearts that differed only in the D locus of class I histocompatibility antigen. The allograft hearts were harvested at 1-3, 5, 7, 14, 28, and 42 days after the transplantation, and the expressions of NF-κB/I-κB and cytokines (TNF-α, INF-γ) were examined in these specimens. The expressions of TNF-α and INF-γ were observed on day 1, peaking on day 5 and 7, respectively. Activated NF-κB (p65) expression was present on the cytoplasm and perinuclear area in the endothelial cells of coronary arteries on day 1. The peak of translocation of NF-κB from cytoplasm to nucleus appeared on day 5 in the endothelial cells, myocytes, and leukocytes within the vessels, and remained elevated until day 42. The I-κB expression gradually increased from day 1 until day 5, but a remarkable decrease was detected on day 7. Our data suggest that the increased expressions of NF-κB/I-κB and cytokines (TNF-α, INF-γ) play an important role in inducing immune responses in the donor allograft heart and hence the blockage of the expressions might be mandatory to avoid a potential graft failure.

Key Words : *NF-kappa B; Cytokines; Heart Transplantation; Coronary Arteriosclerosis; Graft Rejection*

Jeong Ryul Lee, Chul Jun Seok, Joon Seok Kim, Ji Min Chang*, Jeong Wook Seo†

Department of Thoracic and Cardiovascular Surgery, Seoul National University Hospital; Division of Cardiothoracic Surgery*, Seoul Adventist Hospital; Department of Pathology†, Seoul National University Hospital, Seoul, Korea

Received : 26 February 2001
Accepted : 18 May 2001

Address for correspondence
Jeong Ryul Lee, M.D.
Department of Thoracic and Cardiovascular Surgery, Seoul National University Hospital, 28 Yongon-dong, Chongro-gu, Seoul 110-744, Korea
Tel : +82.2-760-2877, Fax : +82.2-765-7117
E-mail : jrl@plaza.snu.ac.kr

*This study was supported by the Seoul National University Hospital Research Fund Ministry of Education of Korea (1995) (C-SL).

INTRODUCTION

Accelerated graft arteriosclerosis (AGAS) of the coronary arteries remains a significant obstacle to the long-term survival of cardiac transplant. Recent studies documented the incidence of AGAS up to 44% in 3 yr after transplantation (1). Although the pathogenesis of AGAS has not been clearly understood yet, there are evidences that it could be resulted from an immunological insult to the coronary arteries associated with histoincompatibilities between the donor and recipient. It is generally believed that AGAS involves a localized cell-mediated immune response initiated by activation of host T-lymphocytes by graft endothelial cells (2, 3). T-cell activation within the vessel wall would cause a local production of cytokine or growth factors that result in an accumulation and proliferation of smooth muscle cells in the intima of graft arteries (4). In addition to the initiating role of major histocompatibility complex (MHC) antigens, inflammatory stimuli such as bacterial lipopoly-saccharide (LPS), interferon-γ (IFN-γ), interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor-α (TNF-α) that are secreted by activated immune cells potently induce leukocyte adhesion molecules to activate endothelial cells and to greatly increase binding of lymphocytes and monocytes. Induction of proinflammatory genes by endothelial cells is often triggered by proinflammatory cytokines such as TNF-α and IL-1, and is largely dependent on the activation of members of the Rel/nuclear factor κB (NF-κB) family of transcription factors (5). The transcription factor NF-κB is a critical regulator of cytokine-inducible gene expression (6). NF-κB is a pleiotropic regulator of many genes involved in immune and inflammatory response including the cytokines, chemokines, growth factors, cell adhesion molecules, immunoreceptors as well as acute-phase proteins. In resting cells, NF-κB resides in an inactive cytosolic form through its interaction with the members of a family of inhibitor proteins, like IκB-α, IκB-β, IκB-ε, or Bcl-3, known as inhibitory κB (I-κB).

The present study was designed to investigate whether these cytokines (IFN-γ, TNF-α) exert their positive effects on the signalling of NF-κB family members by identifying interaction between cytokines and NF-κB in regard to the degree of expression and colocalization. For this, we investigated the early time course of activated NF-κB and cytokines (IFN-γ, TNF-α) in the lesions of transplanted mouse heart allograft tissues. The results provided a correlative data between the temporal and spatial patterns of expres-

sion of the NF-κB/I-κB regulatory system and two κB-dependent cytokine gene products in the development of AGAS lesion in the chronic rejection of transplanted murine heart. In addition, we have demonstrated that this transcription factor is important for the early responses of immune cells (macrophage, lymphocyte) to an immunological insult due to MHC disparity and for cytokine-inducible gene expression in endo-thelial cells of coronary arteries.

MATERIALS AND METHODS

Animal preparation

Adult mice (seven to ten weeks of age) of B10.A and B10.BR strains, weighing 17-22 g, were obtained from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, U.S.A.). The B10.A (H-2Dt^a) and B10.BR (H-2D^k) strains differ only in the D locus of the class I MHC antigen. B10.A mice were used as donors, and B10.BR mice were used as recipients. The mice were housed under conventional conditions and fed a standard diet (Rodent laboratory chows, Cheil Jedang Co., Incheon, Korea) and water ad libitum. After completion of the heterotopic heart transplantation, the mice were allowed to recover with oxygen and local heat, and then transferred into their cages after 24 hr of surgery with a free access to food and water. Each of five recipient mice were sacrificed at 1-3, 5, 7, 14, 28, and 42 days after transplantation.

Heterotopic Heart Transplantation

Intraabdomenal heterotopic heart transplantation was performed with a modification of the technique described by Corry et al. (7). Cardiac allograft from B10.A mice were transplanted into B10.BR mice using standard microsurgical techniques. After anesthesia using 4% chloral hydrate (0.1 mL/20 gm of body weight, intraperitoneal injection) and methoxyflurane (inhalation), a "U"-shaped sternal lid was made and lifted cephalad. Both the right and left superior caval veins were ligated with 5-0 silk. The donor heart was arrested with cold heparinized saline (100 units/mL of saline) delivered via the inferior caval veins. The aorta and main pulmonary artery were then transected. After transection of caval veins distal to the ligatures, the pulmonary veins were ligated en bloc and transected. The donor heart was preserved in 4℃ cold saline solution until the recipient mouse was prepared. Through a midline abdominal skin incision, the recipient's infrarenal abdominal aorta and inferior caval vein were dissected and controlled both proximally and distally with 5-0 silk ligatures. After a longitudinal aortic incision, an end-to-side anastomosis between the transected ends of donor ascending aorta and the recipient abdominal aorta, followed by an end-to-side anastomosis between the transected ends of donor pulmonary artery and the recipient inferior caval vein were performed using 10-0 nylon sutures. Subsequently, the proximal recipient aortic ligature was released first.

Immunohistofluorescence Stain

Tissue specimens were fixed in neutral buffered formalin, embedded in paraffin, and 4-μm sections were placed on glass slides. Paraffin embedded sections were deparaffinized in xylene and hydrated through the standard graded ethanol. Then the tissue was rehydrated in 0.1 M phosphate buffered saline (PBS) for 5 min. Cell-bound antibodies were essentially visualized using a sensitive immunohistochemical technique. For blocking of non-specific binding, sections were incubated in 15 μg/mL of normal horse serum. The sections were then incubated with the primary antibodies of rat anti-mouse IFN-γ, rabbit anti-mouse TNF-α, rabbit anti-mouse p65 (Santa Cruz, U.S.A.), and I-κB/MAD-3 (Santa Cruz, U.S.A.), polyclonal antibody for two hours in a humidified chamber at room temperature. The slides were dipped in PBS in 0.1% Triton X-100 for three min in order to reduce background noise and further washed additional three times with PBS. Then the specimens were incubated with FITC-labeled secondary antibodies (rabbit anti-rat IgG antibodies, swine anti-rabbit IgG antibodies conjugated with FITC (Dako, Denmark) for 30 min at room temperature in the dark. Following subsequent washes in PBS with 0.05% Tween-20, the slides were counterstained with propidium iodide/antifade (Oncor, U.S.A.), and mounted with fluorescence mounting media (Dako, Denmark). Expression of antigen was determined on endothelium of coronary arteries, endocardium, and myocardium. The sections were examined and scored on a scale of 0 to 3 by two investigators in a blind manner (Table 1). Photomicrographs were taken with a fluorescence microscope (Olympus, Japan). All arteries and arterioles larger than 50 mm in diameter were examined and scored in at least 3 sections per graft.

Immunohistochemical Double-staining

On each corresponding day, five mice were sacrificed and freshly dissected heart grafts were removed. Specimens were serially sliced into 4-μm thickness, and preserved in 10%

Table 1. Grading scale of the semi-quantitative method in measuring NF-κB, I-κB, TNF-α, and INF-γ in cardiac tissues

Grade	Extent of immunofluorescent staining
0	Negative
1	Equivocal staining
2	Definite positive staining in intramyocardial arterioles
3	Positive staining in myocytes and interstitium in addition to arterioles

buffered formalin. Tissue specimens were fixed in buffered formalin, embedded in paraffin, and 4-μm sections thereof were placed on glass slides. Paraffin embedded sections were deparaffinized in xylene, hydrated through the standard graded ethanol, and washed 3 times for 5 min each in PBS (pH 7.5). Cell-bound antibodies were visualized using a sensitive immunohistochemical technique. Cell membrane was permeabilized by sequential treatment with 0.2% and 0.5% Triton X-100 in PBS for 10 min each. For blocking of non-specific binding, sections were incubated in 15 μg/mL of normal horse serum. The sections were then incubated with the primary antibodies of rat anti-mouse IFN-γ, rabbit anti-mouse TNF-α, rabbit anti-mouse p65 (Santa Cruz, U.S.A.), polyclonal antibody, and mouse anti-mouse H-2D antibody for 2 hr in a humidified chamber at room temperature. After washing in 1× PBS, the specimens were incubated with FITC-labeled secondary antibodies for rabbit anti-rat IgG antibodies and swine anti-rabbit IgG antibodies (Dako, Denmark), and with Cy™ 3-labeled secondary antibody (Zymed Lab.) of goat anti-mouse IgG antibodies for 1 hr at room temperature in the dark. After subsequent washes in PBS with 0.05% Tween-20, the slides were counterstained with nuclear DAPI and mounted with fluorescence mounting media (Dako, Denmark). Expression of antigen was determined on endothelium of coronary arteries, endocardium, and myocardium. Photomicrographs were taken with a fluorescence microscope (Olympus, Japan).

Confocal Laser Microscopy

To detect the translocation of NF-κB, immunohistofluorescence technique was used with confocal laser microscope (MRC 1024/UV, Biored, U.S.A.) (×600). Re-distribution to the nucleus indicated the activation of transcription factor NF-κB.

Western Blot Analysis

To determine the cytokine IFN-γ in serum samples of the harvested mouse hearts after transplantation, Western blot technique (Towbin et al. 1979; Burnette 1981) was used. Sera diluted with DDW at 1:50 were denatured with Laemmli sample buffer (2× SDS sample buffer) by boiling for 3 min. For Western blot analysis, serum proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane filter at 100 voltage for 1 hr. Following protein transfer, the membrane was washed briefly with 1× TBS. For blocking of non-specific antibody-antigen complex formation, the membrane was immersed in 0.2% non-fat dry milk dissolved in TTBS (150 mmol/L NaCl, 10 mmol/L Tris-HCl, 0.1% Tween 20, pH 7.4) for 1 hr and subsequently shake-incubated for 1 hr at room temperature with a 20 μg/10 mL diluent of primary rat monoclonal antibody against mouse IFN-γ (Pharmingen), followed by washing for 3×5 min in washing buffer. Then the membranes were incubated with alkaline phosphatase-conjugated goat anti-rat IgG at 0.2 μg/mL dilution of blocking buffer for 1 hr. Immunoreactive bands were visualized using Western-Light™ Chemiluminescent Detection System (CSPD® Chemiluminescent Substrate, TROPIX). Immunoreactive bands were visualized using an enhance chemiluminescence kit (Amersham). The membrane was further incubated for 5 min in CSPD Ready-to-Use substrate solution and finally exposed to a film for 10-20 min immediately after the incubation. Quantification was performed by densitometric scanning of bands on the developed film with TINA-2.0 program using Fuji-film BAS (Biological Analyzing System) -2500 read plate and a CCD camera.

Enzyme-linked immunosorbent assay (ELISA)

Plasma samples obtained by recipient mouse heart were left for 30-60 min at room air, and were prepared by centrifugation of whole blood at 3,000 rpm at 4℃ for 10 min. The plasma samples for TNF-α ELISA were sequentially centrifuged at 10,000 rpm at 4℃ for 20 min to remove platelets. The supernatants were transferred to fresh tubes and immediately stored at -70℃ by deep freezer until use. The samples were serially diluted at 1:50 with 0.1% swine serum/PBS and incubated in ELISA modules (*Nunc*) coated with anti-mouse/rat TNF-α monoclonal antibody (Pharmingen) at 4℃ for 24 hr. After washing four times with PBS containing 0.5% Tween 20 (PBS-T), rabbit anti-mouse TNF-α polyclonal antibody (Genzyme) was added as a detection antibody in ELISA modules, followed by incubation at 37℃ for 1 hr. Next, the modules were washed five times with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Vector Lab) for 1 hr at 37℃. Finally, these ELISA modules were washed five times with PBS-T, and a 30% hydrogen peroxide/ ABTS [2, 2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)] substrates were added. The colorimetric reaction was allowed to run for 30 min and was stopped with 1N H_2SO_4. The resulting gradations of yellow color were subtracted from each standard sample. The concentration of TNF-α in each well was determined by extrapolating the measured absorbance at 405 nm on an ELISA reader (Dynatech Lab) to a standard curve.

Statistical Analysis

The mean±standard error values were calculated in each group of mice. ANOVA with single factor analysis was used to determine statistical significance.

RESULTS

All allografts showed palpable contractions at the time of

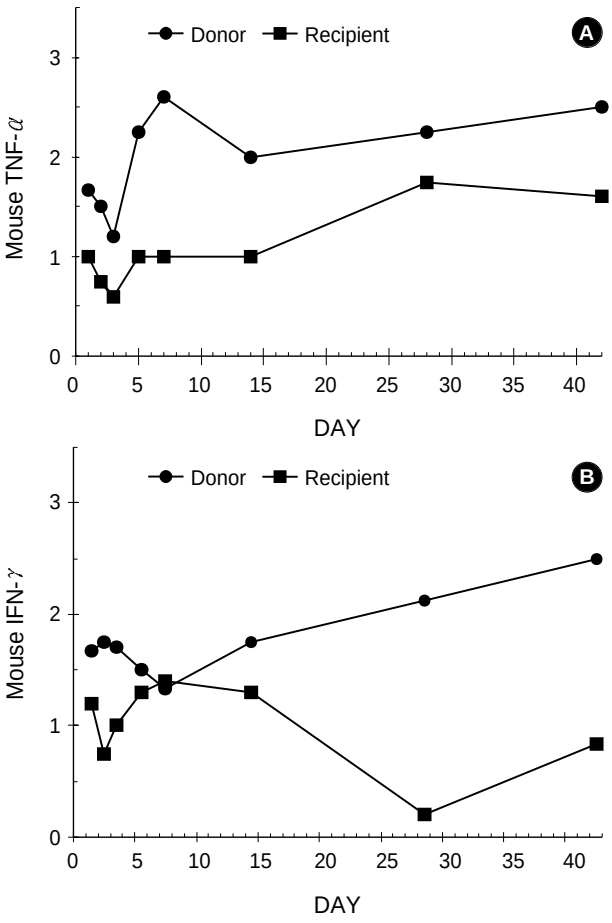

Fig. 1. The expression pattern of TNF-α (A) and IFN-γ (B) in the murine allograft tissue.

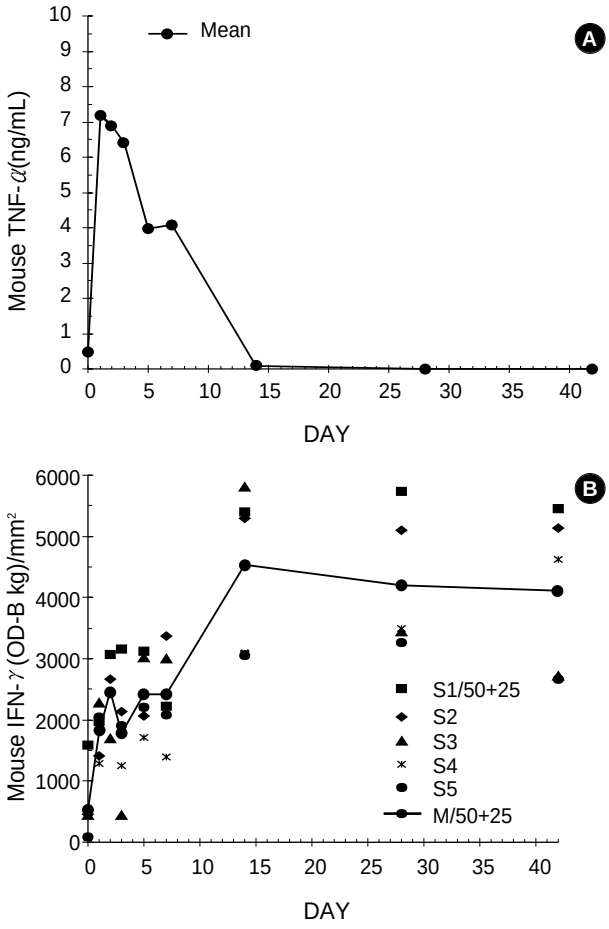

Fig. 2. The serial changes of TNF-α and IFN-γ in allograft serum after transplant. (A) ELISA analysis of TNF-α. (B) Western blot densitometric analysis of IFN-γ.

harvest. Cold ischemic time ranged from 40 to 61 min (mean 49 ± 8 min).

Expression Pattern of Cytokines (TNF-α IFN-γ) in the Allograft Tissue

IFN-γ expression in the cardiac allograft tissues was detected in the activated donor coronary arteries and epicardium with a mean grade of 1.67 ± 0.44 on day 1 after transplantation. The intensity of expression increased gradually after day 7, and remained unchanged thereafter. However, the recipient's tissues showed decreased IFN-γ expression except a mild degree of elevation from day 5 to day 14 (Fig. 1B). TNF-α expression in the donor arteries was detected from day 1 at a mean grade of 1.67 ± 0.48, peaking on day 7, and remained at a mean grade over 2.00 by day 42. By comparison, TNF-α expression in the recipient heart remained at a lower grade throughout the experiment (Fig. 1A).

Level of TNF-α in mouse serum at each time after transplantation were much higher than that in normal control group (B10.BR) during the initial day 1-5 period and thereafter decreased to a lower level than in control (Fig. 2A). Level of IFN-γ from day 1 to day 7 in the graft mouse serum was higher than in normal control group (B10.BR). After day 14, the level of IFN-γ persistently remained strong positive (Fig. 2B). In particular, the level of IFN-γ and TNF-α in early stage was distinctively higher in allograft serum than in normal control group. The peak increases in release of TNF-α and IFN-γ were seen during day 1-5 and after day 14, respectively.

In addition, a time-dependent correlation between cytokines (IFN-γ, TNF-α) and class I MHC expression was revealed by immnunohistochemical procedure for sequential double antigen staining in the donor allograft tissues (Fig. 4). Fig. 4A and B shows a significant correlation in the localizations of TNF-α and class I MHC expression in the epicardium and arteries. Also, Fig. 4C and D shows a significant correlation in the localizations of IFN-γ and class I MHC in the myocardium and the immune cells within the vessel on day 5.

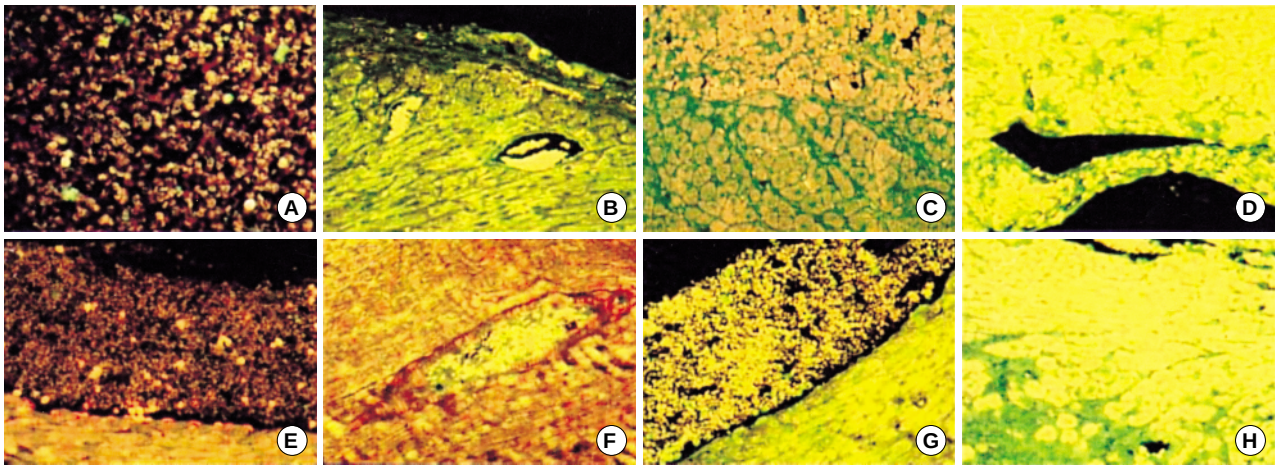

Fig. 3. Expression of activated cytokine (TNF-α, IFN-γ) in cardiac tissues. The TNF-α and IFN-γ expressions are present minimally in the recipient allograft vessel on day 3 (A, E), in the recipient allograft myocardium on day 5 (B, F), and was detected in the vicinity of donor vessel on day 5 (C, G) and in the donor myocardium on day 7 (F, H). Greenish color represents positive staining (A×400, B-H ×200).

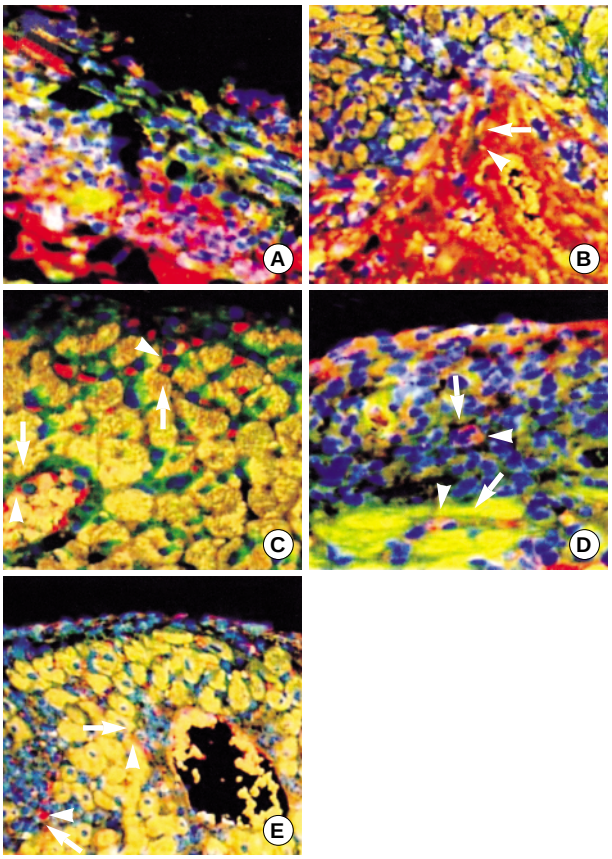

Fig. 4. Colocalization of activated cytokines (TNF-α, IFN-γ) and MHC antigen expressions in the allograft tissues. The green FITC (arrows) indicates a positive expression of cytokines TNF-α (A, B, C) and IFN-γ (D, E). The red Cy3 fluorescence (arrowheads) indicates a positive expression of MHC antigen. Blue DAPI fluorescence is for the nucleus couterstain (×200).

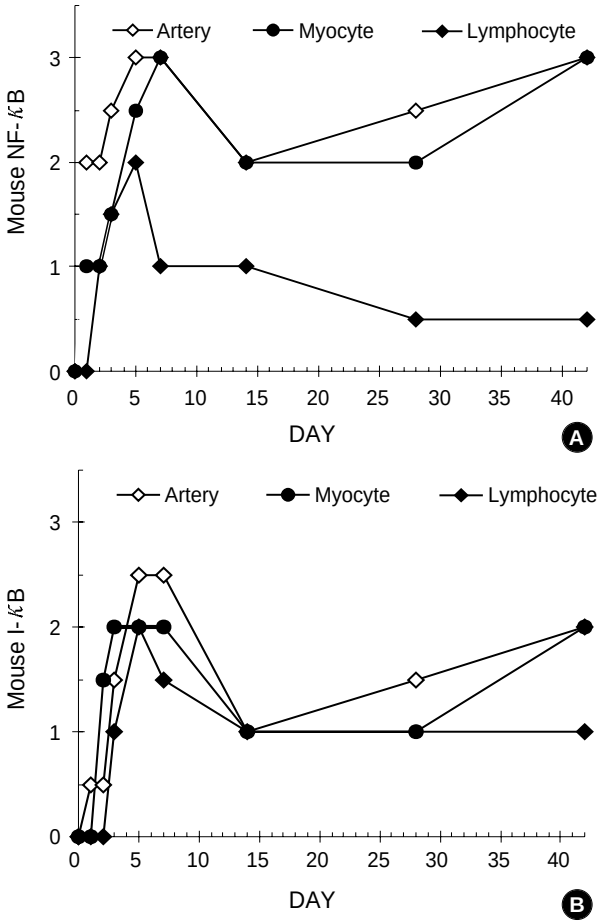

Fig. 5. The expression patterns of transcription factor NF-κB and I-κB in the murine allograft tissues. (A) Temporal and spatial patterns of expression of activated NF-κB and (B) I-κB in the donor artery, myocyte, and lymphocyte after transplantation.

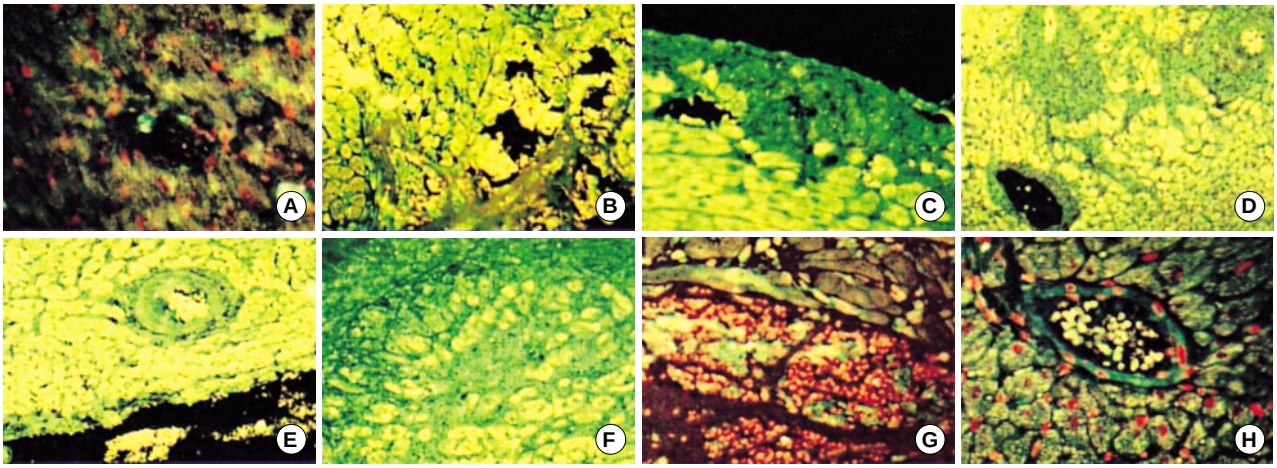

Fig. 6. Expression of activated transcription factor NF-κB and I-κB in the lesion. The expression of activated NF-κB in the AGAS lesion is localized in myocardium on day 1 (A), immune cells within the vessel on day 3 (B), epicardium on day 3 (C), coronary artery on day 5 (D), endothelium and adventitia on day 7 (E), and myocardium on day 7 (F) in an advanced AGAS. The I-κB expression is localized in the immune cells within the vessel on day 3 (G) and coronary artery on day 5 (H). The green fluorescence indicates activated NF-κB and I-κB, which was shown by an indirect immunofluorescence method (×200, G and H ×400).

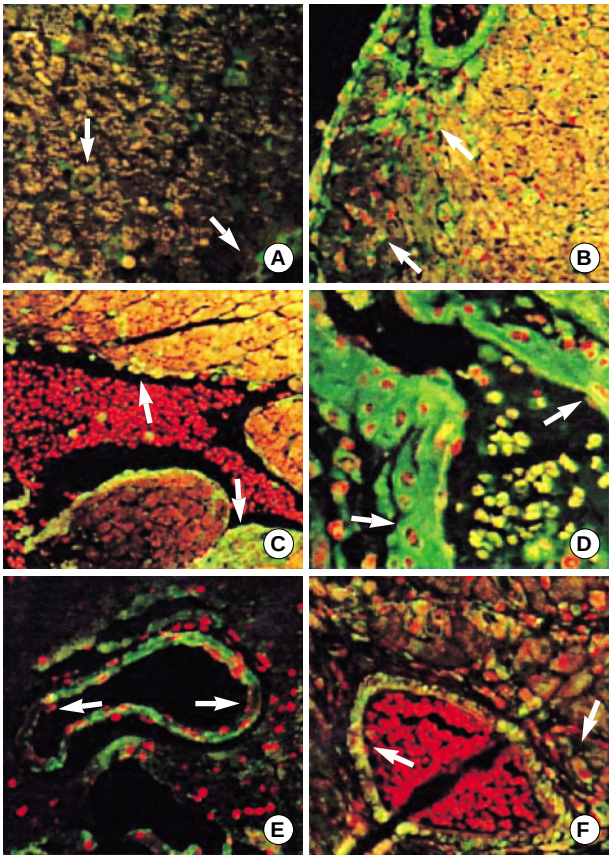

Fig. 7. Translocation of activated transcription factor NF-κB from cytoplasm into nucleus, which was detected by immunofluorescence technique confocal laser microscope. Arrows represent the activated NF-κB in the nucleus (×600).

Expression and Translocation of Activated NF-κB in the Lesion

To detect locational change of p65, a component of NF-κB whose transport is expressed after NF-κB activation, in the lesion, indirect immunofluorescence techniques with confocal laser microscope were employed using the polyclonal antibody against p65. Expression of p65 was not detectable in normal heart of unmanipulated mice, but was detected in endothelial cells and myocardium on day 1 after transplantation. The pattern of location was cytoplasmic and perinuclear. In particular, our immunohistochemical studies showed a partial positive staining for p65 in the nucleus of cardiac endothelial cell, and primarily of immune cells, which are supposed to be the prime target cells for ischemic-reperfusion injury after cardioplegic arrest and reperfusion. On day 3, the location of p65 expression began to change from cytoplasm to nucleus. The change was very little but detectable. This translocation was observed more frequently in epicardium and immune cell within the endocardium than other site of cardiac tissues (Fig. 6B). These cells with translocated p65 were increased from day 3, 5, 7. The peak of translocation ratio and quantitative increase was observed on day 7. However, p65 expression in peripheral blood cells was peaked on day 5 and thereafter remained weakly positive (Fig. 5A). The translocated p65 was predominantly found in the nucleus of endothelial cells in coronary arteries, the significant proportion of myocytes, and frequently in leukocytes within a vessel (Fig. 6). Also, the translocation of p65 could be mostly detected in the immune cells (in particular, macro-phages and a subset of T cells) within the donor vessel in the AGAS (Fig. 6). On day 14, although the translo-

cation of p65 from cytoplasm to nucleus was observed in any sites, cytoplasmic expression was definitely detected. Expression of p65 in nucleus after day 28 was predominantly visualized along with the expression in cytoplasm by immunofluorescence staining, and this expression pattern remained until day 42. NF-κB protein acts as a transcription factor and exerts its effects in the nucleus. Cytoplasmic regulatory mechanisms may modulate NF-κB expression after the translocation. Therefore, our immunohistochemical findings of localization of NF-κB protein in nuclei of endothelial cells and cardiac myocytes may be considered important for the delineation of possible consequences of the increased levels of the protein.

In addition, the present study has shown that coexpression of p65 and class I MHC antigen was localized by immnunohistochemical procedure for sequential double antigen staining in the donor allograft tissues (Fig. 8). The location of p65 and class I MHC antigen expression were excessively relative to interaction in the epicardium and arteries (Fig. 8A). The striking similarity of colocalization of activated NF-κB with class I MHC antigen expression in the endothelial cell indicates that NF-κB is related to the gene expression in the development of AGAS lesion.

Expression of I-κB (N-terminal)

There was no detectable expression of I-κB in normal heart from unmanipulated mice (B10. A) (Fig. 5). In contrast to p65/p50 and p65 for NF-κB, protein for I-κB was found in normal endothelium but the level varies from cell to cell. Increased immunostaining for IκB-α is seen at the wound edge at all late stages along with endothelial hyperplasia. On day 1 after transplantation, there was a trace of expression of I-κB in endothelial cells in coronary artery (CA), which was similar to that in myocardium (M) and leukocytes (L). On day 3, the increased expression of I-κB was found in all three locations (CA, M, and A). The highest level of expression was detected in endothelial cells, and a reaction signal in the nucleus of myocardium was shown (Fig. 6H). The level of I-κB expression was gradually increased on day 5 and 7 (Fig. 6G). However, a remarkable decrease of the expression was detected on day 7 (Fig. 5B).

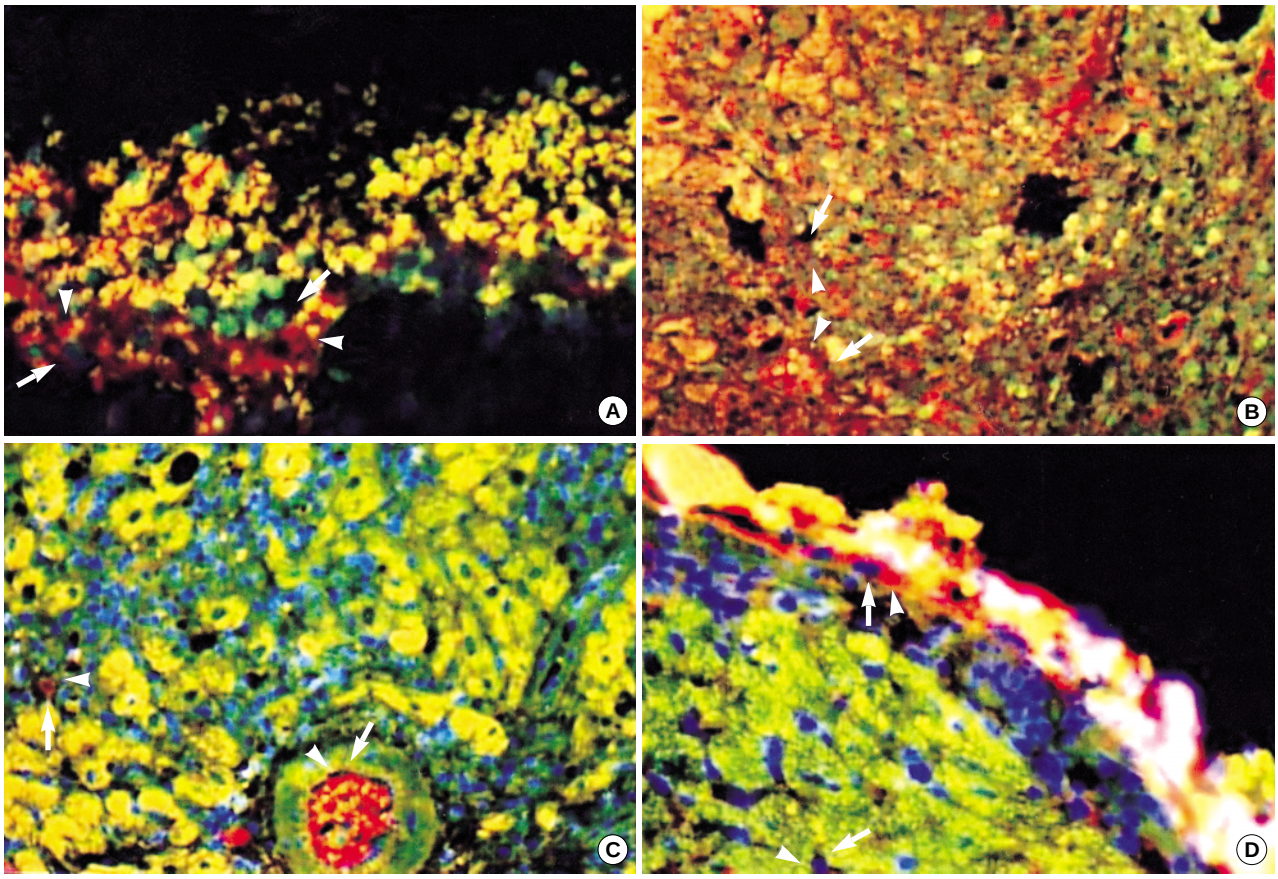

Fig. 8. Colocalization of activated transcription factor NF-κB and MHC antigen in the allograft tissue. The green FITC (arrows) and the red Cy3 fluorescence (arrowheads) indicates a positive expression of activated NF-κB and MHC antigen, respectively. The blue DAPI fluorescence counterstains the nucleus (×200).

DISCUSSION

This study demonstrated the time-course of expressions of transcription factor NF-κB and cytokines (INF-γ, TNF-α) in donor coronary arteries and myocardium in the murine cardiac transplant model with AGAS, and showed the pivotal effects of NF-κB and cytokines on the development of AGAS. We used the mice strains with a minor MHC antigen disparity. The strains (B10.A/B10.BR) differ in the D locus of class I MHC antigen and previously reported as the strain pair with long-term donor heart survival (8, 9) and progressive intimal thickening of coronary arteries (10). Presumably, subclinically sustained immunological insult to the coronary arteries due to the MHC antigen differences between the donor and recipient ultimately results in the development of AGAS (11, 12). Also, previous study confirmed that the incidence of severe post-transplant coronary artery disease in this strain combination was 60% during the 50 postoperative days, which was similar as that in the human cardiac graft arteriosclerosis. In addition, the pathologic features of the lesions were similar to those observed in the human cardiac recipients after long survival, which consist mainly of vascular smooth cell growth and limited inflammatory cell infiltrations primarily of macrophages (13, 14). Despite the observation of chronic vascular rejection by numerous investigators, little is known about the early time-dependent course of the immunological events including immune cell infiltration, cytokines levels, and MHC antigens. However, it is clear that either class I or class II alloantigens plays an important role as an inducer of the rejection of vascularized heart allograft even though MHC disparity is not consistently related to the kinetics or rate of graft rejection and the patterns of MHC expression among strains. Our previous studies have shown that the class I MHC antigen (H-2D) expression of the donor tissues (endothelial and medial cells of coronary arteries) was highest on the 7th post-transplant day and then remained elevated, whereas that in the recipient tissues was only partly present on infiltrating monocytes. Moreover, class II MHC antigen was not detected at any point of time (15). The upregulation of class I MHC antigen activity by MHC disparity may stimulate T cell immune activity and subsequently cytokine production. Thereafter, circulating T cells that encounter endothelial cells bearing foreign class I MHC molecules produce cytokines that in turn modulate MHC antigen expression of endothelial cells and macrophage activation. A subset of increased activated T lymphocytes infiltrates to the endothelial cells and induces an inflammatory response to the endothelial cells in the early period of development of arteiosclerotic lesions. Therefore, the infiltration-associated inflammatory cell subsets induce activation and adherence of macrophages, further accelerating the infiltration to the allograft tissues (16). We also observed a large number of inflammatory cells adherent to the endothelium as well as adventitia. In the earlier stage of intimal proliferation, clusters of subendothelial lymphocytes were seen. In the vicinity of the endothelial lesion, inflammatory cells were recruited and accumulated in the subendothelial space. A mild to moderate degree of adventitial inflammatory cell infiltration was observed even on day 7. This advantitial lymphocytes were commonly seen in epicardial vessels and macrophages interspersed among them (17).

We analysed the expression pattern of NF-κB as a transcription mediator that activates T lymphocyte to secrete cytokines (IFN-γ, TNF-α). The high level of NF-κB expression was observed in the immune cells within the coronary arteries in the early period (Fig. 5, 6A), concomitantly with a high level of class I MHC antigen expression (Fig. 8). These data might suggest the synergistic induction of cytokines by NF-κB and class I MHC antigen in activated immune cells, a distinctive interrelation between these two molecules. Furthermore, we confirmed in a sequential manner that the expression of cytokines (IFN-γ, TNF-α) in the inflammatory cells and of NF-κB was upregulated in the lesion. These inflammatory stimuli of TNF-α was observed as a high level of its expression primarily in serum in the early stage of post-transplantation (Fig. 2A), and its expression in donor tissues was significantly increased from day 5 and on (Fig. 1A). These patterns show a temporal and spatial correlation between TNF-α stimulation and activated NF-κB expression. IFN-γ was released from the activated immune and endothelial cells, and its expression was visualized at a similarly low level in the serum and tissues in early post-transplantation period, and then gradually increased, and remained elevated until day 42 (Fig. 1B). However, the level of TNF-α was significantly high in the serum during day 3-5, and showed a continuous increase therafter. The localization of TNF-α expression was observed similar to that of the strong NF-κB-positive cells. We also found a strong correlation between the activated NF-κB-modulated immune system and the cytokines (IFN-γ, TNF-α) expression as a markers of inflammatory response.

Although the pathogenesis of AGAS is not clearly understood yet, our data indicate that T cell activation within the vascular lesion can cause a local production of cytokines or growth factors, which subsequently results in an accumulation and proliferation of intima and smooth muscle cells of graft arteries. Then, the intimal thickening, smooth muscle cell proliferation, and migration into the intima, which are collectively the major natures of α-actin-positive smooth muscle cells, progress in donor coronary arteries and ultimately develop the arteiosclerotic lesion (18). In addition, the cytokines and growth factors that regulate migration and proliferation of these smooth muscle cells were secreted from the injured intima and released from infiltrating inflammatory cells. The early stages of transplant arteriosclerosis may involve the immune responses directed to the graft endothelium, which may stimulate intimal and smooth muscle cell

proliferation in response to repeated endothelial injury (4, 16).

In the present study, we have sequentially examined a time-dependent interrelation between the expression of cytokines as NF-κB targets and that of transcription factor NF-κB as a regulator of cytokines production in the coronary arteries to characterize the evolution of AGAS lesions. The immune cells and cytokines in these heterotopic cardiac transplant have relevance to development of allograft vasculopathy (16), and the alloantigens-expressed class I MHC antigen induce activation and proliferation of cytolytic T cell (CTLs, $CD8^+$). Activated NF-κB was found in the thickened intima/media, in the myocardial lesion, and in both T cells and macrophages (Fig. 5, 6). These observations suggest that transcription factors, perhaps in association with cytokine secretion by infiltrating T lymphocytes, play an important role in the development of the arteriosclerotic lesions. The cytokines secreted by these activated $CD8^+$ lymphocytes promote the adhesion of MHC-restricted T cells to antigen-presenting cells (APCs) or to target cells by virtue of their specific affinity toward MHC molecules, and participate in the early signal transduction events that occur upon T cell recognition of peptide-MHC complexes on APCs. Activated endothelial cells and infiltrating mononuclear cells secrete growth factors, which promote the proliferation of smooth muscle cells, and ultimately lead to an intimal thickening. As shown in Fig. 4, the data suggest that colocalization of a high-frequency donor class I MHC antigen and cytokines (IFN-γ, TNF-α) expression might play a triggering role in the intimal thickening during the development of AGAS. Antigen presentation and IL-1 production by macrophages result in activation and proliferation of T-helper cells, and subsequently the release of IL-2 and other cytokines by the activated T-helper cells results in the recruitment, activation, and proliferation of cytotoxic T cells, suppressor T cells, natural killer cells, and B cells. T-helper cells may play an important role in immune-mediated vascular pathology because they can be activated by MHC class I antigens expressed on the vascular endothelial cells and smooth muscle cells. Activated T-cells can also produce cytokines (IFN-γ, TNF-α), which stimulate expression of adhesion molecules, resulting in further recruitment of immune cells to the vascular endothelium. The secretion of a variety of cytokines (IFN-γ, TNF-α, and IL-6) was induced by the activated cytolytic T cells ($CD8^+$) (19), and in particular, increased IFN-γ further accelerates activation of macrophages (2, 20). In addition, IFN-γ activates macrophages, which in turn release an increased quantities of growth factors. Fig. 3 shows the time-dependent analysis of IFN-γ expression in arteries and myocardium during the development of AGAS. IFN-γ, a cytokine produced by certain T cells subset and natural killer cells, would presumably play an initiative role in this sequence by directly activating macrophages and by enhancing the expressions of MHC products, other components of the antigen presentation pathway, and adhesion molecules. Also, the cytokines can be activated by the upregulation of transcription factors, such as NF-κB. VCAM-1 and ICAM-1 expression induces an infiltration of inflammatory cells to target cells on the vascular endothelium, and further activates class I and II MHC antigens expression (12).

For the infiltration of these inflammatory cells into a target site and for the release cytokines by these infiltrating cells, recent evidence suggests that transcription factor NF-κB, a pleiotropic regulator that can modulate a numerous genes, is related to the expression of adhesion molecules in activated endothelium and to cytokine (IFN-γ, TNF-α) production involved in immune or inflammatory response. Inflammatory mediators, including LPS, IFN-γ, IL-1, and TNF-α, strongly induce ICAM-1 in a variety of tissues and greatly increase the binding of lymphocytes and monocytes. Activated endothelial cells vary as to which cytokines are capable of inducing ICAM-1 expression, and the type of mediators released may therefore help regulate different patterns of cell localization induced by inflammatory stimuli. The production of inflammatory mediators (primarily of IFN-γ, TNF-α) by lymphocytes and macrophages plays an important role in increasing the ability of endothelial cells to express leukocyte-specific adhesion molecules, to secrete inflammatory cytokines, and to facilitate the extravascular movement of inflammatory cells. Therefore, the present study shows that the increase of cytokines (IFN-γ, TNF-α) is related with the development of AGAS on day 7. In our recent model, vascular adhesion molecules, such as ICAM-1 and VCAM-1, were shown regulate the adhesion of leukocytes to vascular endothelium, leading to a migration of cells into the target tissue and play an important role in the graft rejection. The ICAM-1 and VCAM-1 expressions in post-transplant donor heart were increased in the very early stage and remained elevated throughout the study. ICAM-1 and VCAM-1 expressions in epicardial coronary endothelium were peaked on day 7 and 5, respectively, and remained unchanged until day 42. However, very low expressions thereof were noted in the recipient's coronary artery (17). The subsequent release of cytokines enhances adhesion and migration of inflammatory cells through endothelial cells into the tissue. The cytokines within the lesion cause upregulation of class I MHC antigen and vascular adhesion molecules, which make the endothelium a target for cytotoxic damage by T cell ($CD8^+$) activation. The patterns of transcription factor activation and ICAM-1 and VCAM-1 expression were immediately seen adjacent to the advanced lesion. These suggest that the localized activation of the NF-κB system in the AGAS induces the expression of a leukocyte adhesion molecule. We have shown that the patterns of cytokine (IFN-γ, TNF-α) expressions induced by the upregulation of NF-κB and of adhesion molecule expression were somewhat similar in the lesions. These observations suggest that the activated transcription factor NF-κB modulates both adhesion molecule and cytokines (IFN-γ, TNF-α) expression, and

conversely NF-κB is stimulated by the cytokines (IFN-γ, TNF-α) released from the CTLs cells. These findings provide a correlative implication between the temporal and spatial patterns of expression of the NF-κB/I-κB regulatory system and two κB-dependent cell adhe-sion molecule genes in the development of AGAS. In addition, these results presumably provide an indirect evidence for the inflammatory response caused by MHC disparity in the AGAS lesion, and therefore suggest the need for blocking this system in lesions with less advanced arteriosclerosis. Therefore, understanding of the function of NF-κB would be fundamental to the study of cytokines and cytokine-mediated inflammation, and may provide a novel therapeutic strategies for the treatment and prevention of various inflammatory diseases (21, 22). To minimize the inflammatory response associated with organ xenograft rejection, it seems mandatory to inhibit NF-κB that has been shown to play a pivotal role in the induction of the proinflammatory genes associated with endothelial cell activation (23).

REFERENCES

1. Uretsky BF, Murali S, Reddy PS, Rabin B, Lee A, Griffith BP, Hardesty RL, Trento A, Bahnson HT. *Development of coronary artery disease in cardiac transplant patients receiving immunosuppressive therapy with cyclosporine and prednisone. Circulation 1987; 76: 827-34.*
2. Libby P, Salomon RN, Payne DD, Schoen FJ, Pober JS. *Functions of vascular wall cells related to development of transplantation-associated coronary arteriosclerosis. Transplant Proc 1989; 21: 3677-84.*
3. Hosenpud JD, Shipley GD, Wagner CR. *Cardiac allograft vasculopathy: Current concepts, recent developments, and future direction. J Heart Lung Transplant 1992; 11: 9-23.*
4. Hruban RH, Beschorner WE, Baumgartner WA, Augustine SM, Ren H, Reitz BA, Hutchins GM. *Accelerated arteriosclerosis in heart transplants recipients is associated with a T-lymphocyte-mediated endothelialitis. Am J Pathol 1990; 137: 871-82.*
5. Read MA, Whitley MZ, Williams AJ, Collins T. *NF-kappa B and I kappa B alpha: an inducible regulatory system in endothelial activation. J Exp Med 1994; 179: 503-12.*
6. Baeuerle PA, Henkel T. *Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994; 12: 141-79.*
7. Corry RJ, winn HJ, Russell PS. *Primary vascularized allografts of hearts in mice. The role of H-2D, H-2K and non-H-2 antigens in rejection. Transplantation 1973; 16: 343-50.*
8. Burdick JF, Clow LW. *Rejection of murine cardiac allografts. II. Evidence that splenocytes bearing Lyt2 inhibit responsiveness in long-term heart graft recipients. Transplantation 1987; 43: 509-14.*
9. Russell PS, Chase CM, Winn HJ, Colvin RB. *Coronary atherosclerosis in transplanted mouse hearts. I. Time course and immunogenetic and immunopathological considerations. Am J Pathol 1994; 144: 260-74.*
10. Ardehali A, Billingsley A, Laks H, Drinkwater DC Jr, Sorensen TJ, Drake TA. *Experimental cardiac allograft vasculopathy in mice. J Heart Lung Transplant 1993; 12: 730-5.*
11. Foegh ML. *Chronic rejection-graft arteriosclerosis. Transplant Proc 1990; 22: 119-22.*
12. Boehm U, Klamp T, Groot M, Howard JC. *Cellular responses to interferon-gamma. Annu Rev Immunol 1997; 15: 749-95.*
13. Pucci AM, Forbes RD, Billingham ME. *1990. Pathologic features in long-term cardiac allografts. J Heart Transplant 9: 339-45.*
14. Billingham ME. *Histopathology of graft coronary disease. J Heart Lung Transplant 1992; 11: S38-44.*
15. Lee JR, Lim HG, Seo JW. *Early time course of immunopathologic changes in coronary arteries of heterotopically transplanted mouse heart. J Korean Thor Cardiovasc Surg 1998; 31: 1119-26.*
16. Cramer DV, Wu GD, Chapman FA, Cajulis E, Wang HK, Makowka L. *Lymphocytic subsets and histopathologic changes associated with the development of heart transplant arteriosclerosis. J Heart Lung Transplant 1992; 11: 458-66.*
17. Lee JR, Huh JH, Seo JW, Suk CJ, Jeong HM, Kim EK. *Time-dependent expression of ICAM-1 VCAM-1 on coronaries of the heterotopically transplanted mouse heart. J Korean Med Sci 1999; 14: 245-52.*
18. Cramer DV, Wu GD, Chapman FA, Cajulis E, Wang HK, Makowka L. *Lymphocytic subsets and histopathologic changes associated with the development of heart transplant arteriosclerosis. J Heart Lung Transplant 1992; 11: 458-65.*
19. Fong TA, Mosmann TR. *Alloreactive murine CD8+ T cell clones secrete the Th1 pattern of cytokines. J Immunol 1990; 144: 1744-52.*
20. Nagano H, Libby P, Taylor MK, Hasegawa S, Stinn JL, Becker G, Tilney NL, Mitchell RN. *Coronary arteriosclerosis after T-cell-mediated injury in transplanted mouse hearts: role of interferon-gamma. Am J Pathol 1998; 152: 1187-97.*
21. Soares MP, Muniappan A, Kaczmarek E, Koziak K, Wrighton CJ, Steinhauslin F, Ferran C, Winkler H, Bach FH, Anrather J. *Adenovirus-mediated expression of a dominant negative mutant of p65/RelA inhibits proinflammatory gene expression in endothelial cells without sensitizing to apoptosis. J Immunol 1998; 161: 4572-82.*
22. Chen F, Castranova V, Shi X, Demers LM. *New insights into the role of nuclear factor-kappa B, a ubiquitous transcription factor in the initiation of diseases. Clin Chem 1999; 45: 7-17.*
23. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. *Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB J 1995; 9: 899-909.*