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ORIGINAL ARTICLE

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Expression of fetal isoforms of actin after transplantation injury

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Abstract Trauma and injury to transplanted organs in the early post-transplant period are significant factors that affect long-term graft survival. Fetal isoforms of actin are integral members of the immediate early gene family and are expressed in response to free radical injury. We therefore studied actin gene expression in heart transplantation to determine if reperfusion injury activates fetal isoforms of actins. Heterotopic cardiac transplantations were performed in mice. mRNA was extracted from allo- and isografted hearts as well as from normal hearts and spleen. Northern hybridization with actin cDNA to α and β/γ actin mRNA was performed and analyzed by densitometry. The β/γ actin gene expression in the transplanted hearts was found to be significantly elevated within 48 h after transplantation. Analysis of β/γ actin gene expression in isografts substantiates the possibility of de novo increase in actin expression.Our studies demonstrate for the first time that fetal isoforms of actin are induced in the allograft heart after transplantation.

Keywords MHC · Gene expression · Early immediate genes · Actin gene expression · Mouse heart grafts

Introduction

Trauma and injury to transplanted organs in the early post-transplant period is increasingly being recognized as a factor in long-term graft survival. Of the early post-transplant events, reperfusion injury is associated with the most deleterious long-term consequences [3, 23]. Reperfusion injury causes organ dysfunction in the short run; in the long run it seems to predispose transplanted organs to chronic rejections [29]. The effector mechanisms of reperfusion injury include generation of free radicals, commonly of the oxy radical family, which lead the brunt of the attack. Since these radicals are deleterious to the transplanted organs, their generation has received intense scrutiny. Interventions to limit the generation of the radicals suggested by these studies are beginning to show beneficial effects on long-term survival of transplanted organs [16, 26].

Although there is a fair amount of knowledge on the generation of these radicals, their mode of effecting injury to transplanted organs is not well understood. These radicals appear to induce the expression of genes encoding heat shock proteins [6] and those that are members of the family of immediate early genes [31]. The genes in the latter family are particularly interesting because they encode genes for several transcription factors which have the potential to activate multiple genes, including immunologically important ones like IL-2 [22].

Of even greater interest is that the immediate early (IE) gene family also includes genes encoding structural proteins [13], which can exert a global effect on injured

cells. A particularly relevant example of the latter is genes that encode various isoforms of actin that are induced in vitro by free radical injury [5]. Of the three isoforms of actin, the β and γ isoforms which are found in the developmental stages of cardiogenesis [8, 18, 19, 27] appear to be more commonly induced by reperfusion than the α isoform, which is the predominant isoform in the adult heart. Activation of the actin genes in the cardiac myocytes by reperfusion injury is, therefore, of potential significance in cardiac transplantation from a dual perspective. Firstly, being part of the immediate early genes, these genes could induce a cascade of gene activation that increases the expression of immunologically important genes, including those for major histocompatibility complex (MHC) class II antigens. Also, from a structural perspective, the induction of the fetal isoforms of actin in the adult transplanted heart might provide clues for the cardiac dysfunction that can complicate heart transplantation. Previous studies in humans have attempted to address these issues but have been limited by the availability of specimens and the many variables that commonly confound clinical studies [14, 28].

Given the dual importance of the induction of actin genes in heart transplantation, we studied their expression in a murine model of heterotopic heart transplantation [4, 12]. Since our interest was in the early post-transplant period, we studied the kinetics of expression of the three isoforms of actin in the 1st week after transplantation. Our objectives were to understand the relation of actin expression to reperfusion injury and also to acute rejection. We therefore studied actin gene expression in isografts for the first objective and in allografts to fulfill the second objective. We found that the β/γ isoforms are induced de novo in the immediate post-transplant period, suggesting strongly that these IE genes are induced in response to reperfusion injury and participate in the transplanted heart's response to injury.

Materials and methods

Mouse heart transplantation

The animal care and procedures used in this study were approved and performed in accordance with the institutional animal care committee and according to the U.S. Department of Agriculture Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals.

Allografts were performed between congenic resistant strains of mice. Hearts removed from B10.BR(H-2^k) mice were transplanted heterotypically to B10.D2(H-2^d) mice as described [4]. Briefly, donors were heparinized and their hearts excised rapidly and immediately immersed in chilled lactated Ringer's solution. These hearts were then anastomosed to the abdominal aorta and inferior vena cava of the recipients. All transplanted animals survived the operation. The abdominal heartbeat was palpated to monitor the functional status of the transplanted heart and graded on a score of 0 (no heartbeat) to 4+ (strong heartbeat). All transplanted hearts had 4+ heartbeats the day following surgery. Hearts thus transplanted were excised sequentially from the 2nd to the 6th days after transplantation. Sections were cut through the ventricles for histology and the rest of the heart tissue was snap-frozen until further use. Isografts were performed between B10.BR (donor) and B10.BR (recipient) using similar surgical technique. All isografts functioned well, as did the allografts in the early days after grafting. The allografts later in the course had diminished function which coincided with rejection.

mRNA extraction from transplanted hearts

mRNA was extracted from allo- and isografted hearts as well as from normal hearts and spleens of naive B10.BR mice as described [33]. Briefly, tissues were homogenized in guanidium isothiocyanate and the homogenate ultracentrifuged on a cesium chloride gradient at 35,000 rpm at 4°C for 18 h. The supernatant was carefully removed to prevent DNA contamination of the pellet. The pellet was solubilized in tris ethylenediaminetetra-acetate (TE) buffer and the optical density determined at 260/280 nM. RNA yielding 260/280 ratios of 1.8 or greater was used for further experiments. Further isolation of polyadenylated (Poly A) RNA from total cellular RNA was done by affinity chromatography of total cellular RNA using an oligo (dt)-cellulose type 7 column (Pharmacia). Generally, 3–7% poly A RNA were recovered from the total cellular RNA. All laboratory ware used in RNA experiments was autoclaved to prevent contamination with RNAase.

Northern hybridization with actin cDNA

A 2.1 Kb BamHI fragment of the actin cDNA [10] was radiolabeled with ^{32}P dCTP using nick translation reaction. This cDNA probe hybridizes with β and α actin mRNA in addition to the actin mRNA (Fig. 1).

mRNA from the hearts was size-fractionated by electrophoresis in denaturing 1% agarose and formaldehyde gel and transferred to Genescreen plus membranes by Northern transfer. These filters were baked at 80°C for 2 h before hybridization with the actin cDNA probe. They were then prehybridized in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate, and 100 μm/ml salmon sperm DNA for 1 h at 42°C and then hybridized with radio labeled cDNA probe in the same buffer for 20 h at 42°C. The filters were washed twice in 2XSSC at room temperature for 5 min, then washed twice in 2XSSC and 1% SDS at 60°C for 30 min, and again washed twice in 0.1XSSC at room temperature for 30 min. The filters were then autoradiographed using image intensifier and Kodak X-Omat AR film.

Histopathologic evaluation of the lymphocytic infiltrate in the transplanted hearts

Representative sections through both the ventricles were stained with hematoxylin and eosin. The lymphocytic infiltrate in these hearts was graded on a score of 0 to 3 as follows: grade 0 (no lymphocytic infiltrate), grade 1 (mild infiltrate, limited to one or less areas per high power field [HPF]), grade 2 (more than one area of moderate infiltrate per HPF, but areas not coalescent), and grade 3 (coalescing areas of dense lymphocytic infiltrate in the graft). Myocardial necrosis when noted was taken to represent moderate to severe rejection.

Quantitation of actin gene transcription in allografted hearts

The autoradiographic bands obtained by hybridization of radiolabeled actin cDNA to α and β/γ actin mRNA in normal hearts,

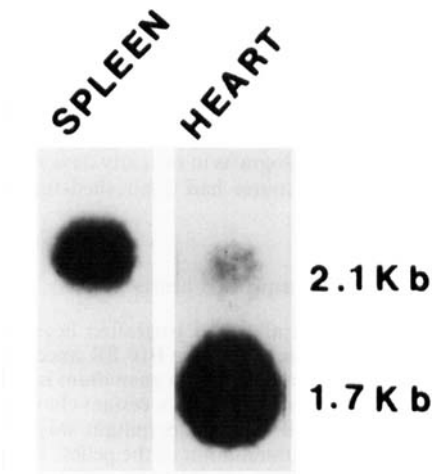

Fig. 1. Actin isoforms in normal adult hearts: comparison with the isoforms in the spleen. The mRNA for β/γ isoforms is distinguished from that for the ∞ isoform on the basis of its size

spleens, and transplanted hearts were analyzed by densitometry. For each time point after transplantation, at least two and in some instances three transplanted hearts were analyzed to obtain average values for the α actin band and similarly for the β/γ actin band. Since α actin expression appeared unchanged, we used the ratio of the intensity of β/γ actin band to the intensity of the α actin band as an indicator of β/γ actin mRNA levels in the hearts. The statistical significance of the difference between the β/γ:α actin ratio in allografts and isografts was determined using Student's *t*-test.

Results

Grading of lymphocytic infiltration in iso- and allografted hearts

Our aim in these experiments was to determine unambiguously if the β/γ actin gene was induced in the cardiac tissue. Since lymphocytes also express β/γ actin, it was important to determine if the iso- and allografts that were analyzed for β/γ actin mRNA contained infiltrating lymphocytes. By semiquantitative grading, we determined that lymphocytic infiltrations in allografts was maximum on the 6th day after transplantation (Fig. 3). In the intermediate period (3rd to 5th post-transplant days), the lymphocytic infiltration was variable. In the early post-transplant period (within 48 h), there was minimal lymphocytic infiltration in the allografts. Most isografts, as expected, revealed minimal (grade 1) lymphocytic infiltration, which had virtually disappeared by the 6th post-transplant day.

Early induction of β/γ actin in transplanted hearts

In the hearts from normal B10.Br mice, mRNA for the α isoform of actin predominated. This finding was similar to that reported by others [11, 13]. The ratio of β/γ actin to α actin in these hearts was 0.2, demonstrating the low level of β/γ mRNA in normal hearts. In contrast to the normal hearts, the β/γ actin gene expression in the transplanted hearts was found to be significantly elevated within 48 h of the transplant procedure (Fig. 2, Fig. 3). This early expression of the β/γ actin gene in the allografts was presumably in the cardiac tissue and not in infiltrating lymphocytes, because histology of the hearts revealed few lymphocytes in this early post-transplant period. There was, however, a late peak in β/γ actin gene expression in allografts which coincided well with lymphocytic infiltration (Fig. 3). Thus, the β/γ actin gene expression in allografts followed a biphasic pattern in which the early peak was either from normal β/γ actin expression in the infiltrating cells or from de novo β/γ transcription in myocytes.

Analysis of β/γ actin expression in isografts appeared to substantiate the latter possibility of de novo increasing of action expression. These isografted hearts were subjected only to ischemia reperfusion but not immunologic attack, as evidenced by a lack of lymphocytic infiltration on all days studied (data not shown). Thus, any elevation of β/γ actin expression in the isografted hearts was very likely to be from cardiac tissue and not from infiltrating lymphocytes. These isografts indeed revealed a high β/γ actin expression within 48 h of the transplant procedure. This induction of β/γ actin did not reach the levels seen in allografts nor was there a biphasic pattern that was seen in the allografts (Fig. 4). This lack of variation probably reflects the lack of infiltrating cells, which could contribute to the total level of β/γ actin mRNA. Thus, the isografts provided further support to the notion that β/γ actin mRNA is induced in the transplanted cardiac tissues as a consequence of anoxia and/or reperfusion injury.

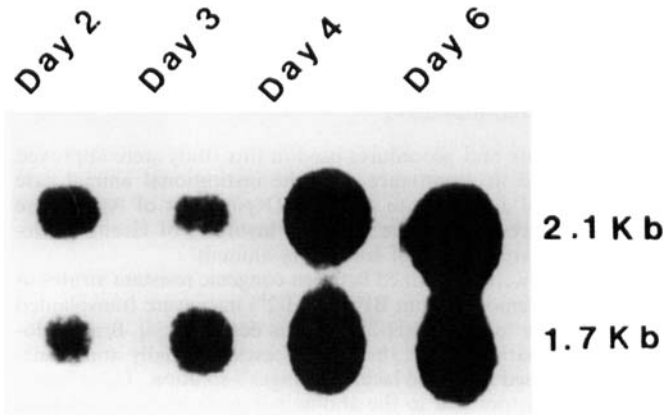

Fig. 2. Actin gene expression in mouse heart allografts: the normal predominance of α actin in RNA is replaced by a predominance of β/γ actin mRNA in the transplanted hearts

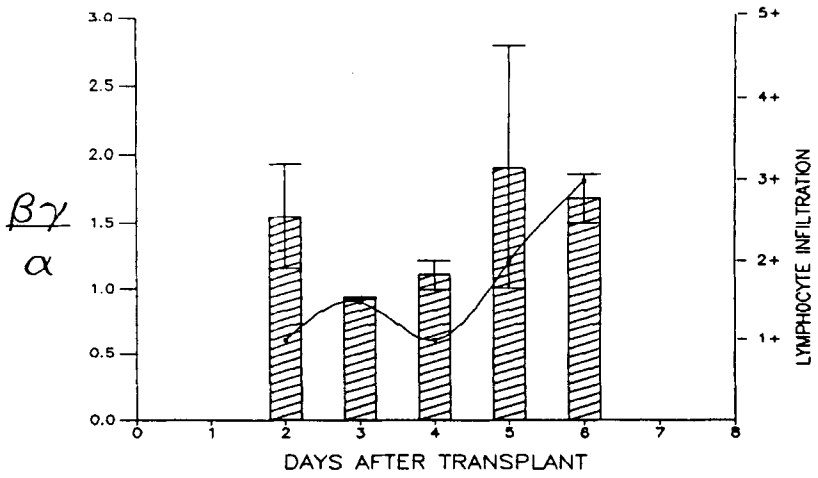

Fig. 3. Relation of lymphocytic infiltration to the β/α actin expression in mouse heart allografts: lymphocytic infiltration in the allografts was semiquantitatively assessed to exclude the probability that the early (day 2) peak in the allograft was from the β/α actin mRNA in the infiltrating lymphocytes. The late peak of β/α actin expression coincides with lymphocytic infiltration, but the early peak does not, which is consistent with the data in Fig. 2

Discussion

Our experiments demonstrate for the first time in a well-defined animal model that the fetal isoforms of cardiac muscle actin are induced in the heart after transplantation. These fetal (β/γ) isoforms of actin are not expressed in normal hearts in adult animals, although they predominate during embryonic development. In this respect, our findings provide an in vivo demonstration that injury to the myocardium initiates the activation of the immediate early genes that have been shown in vitro to respond to injury mediated by free radicals and other noxious agents. The findings gain more relevance because actin is a major structural protein in cardiac muscle.

Prior studies of actin's role as a marker of injury after heart transplantation have had inconclusive results. In most of these involving human heart transplantation, the analysis has been complicated by confounding variables common to clinical experimentation. As a result, it is unclear whether the expression of actin in transplanted hearts is a result of cardiac hypertrophy or injury due to rejection [14, 28]. Our present studies of heart transplantation in the mouse have the advantage of controlling these variables, as previously demonstrated in our studies of the kinetics of MHC class II genes and several other cytokines in this mouse model [12, 33].

Even in the defined model of mouse heart transplantation, it can be difficult to distinguish gene expression in the cells of donor origin from those in cells of host origin. In the case of MHC class II genes, however, we took advantage of allelic differences between the donor and recipient class II genes to construct specific oligonucleotide probes [33]. In the present experiments, we utilized a common cDNA probe [10] but were able to distinguish the different isoforms of actin based on differences in sizes of their corresponding mRNA [27]. In these experiments, we did not study the protein

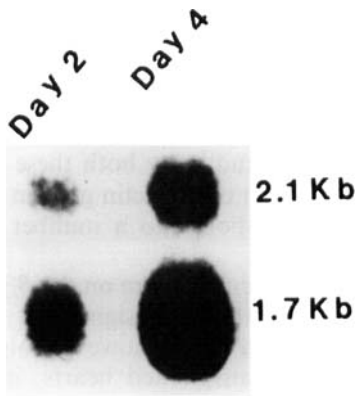

Fig. 4. Actin gene expression in normal heart isografts: a pattern similar to that in allografts is seen in the isografts, although the β/α expression is not as intense as that in allografts. This probably reflects the almost complete lack of infiltrating cells in the isografts

expression but, given the homogeneity of cardiac muscle and the lack of infiltrating cells that could confound the analysis, it is very likely that the actin gene expression is in the transplanted cardiac muscle. Future experiments will confirm this conclusion.

Prior studies in this model have focused on the expression of genes thought to be a direct immunological consequence and only incidentally upon genes induced by trauma. In addition to studies of MHC class II and cytokine genes by others and ourselves, several studies have described expression of co-stimulatory [30] and adhesion molecules in transplanted mouse hearts [9, 22]. The number of studies considering non-immunological genes in this model remains small. Given the recent demonstration of non-immunological factors contributing to long-term graft survival [26], expression of genes responsive to such factors is of increasing interest. In this regard, the mouse model is important to both immunologically important studies (e.g., transplantation) and nonimmunological studies (e.g., trauma).

Reperfusion injury is the most likely mechanism that sets in motion the inflammatory events in the allograft that induce the expression of β/γ actin and other intermediate early genes. Free radicals causing injury in transplanted organs can also induce actin and other intermediate early genes in vitro [5]. Although we presume that this injury induced the expression of the β/γ actins in the murine heart grafts, it is possible that other inducers of these important myocardial proteins also participated in their induction.

Beta actins, similar to many other immediate early genes, have a serum responsive element (SRE) in their promoter region. These SREs mediate the response of the immediate early genes to growth factors and other mitogenic stimuli [15]. Indeed, when the induction pattern of several immediate early genes by IL-3 and PMA was studied, β actin was found to be induced by both mitogenic stimuli, while most other immediate early genes were induced by only one or the other stimulus [17]. Recent studies by Onyia et al. [21] have shown that the β actin promoter region contains CCAAT and CCArGG boxes responsive to several hormones, including insulin. The studies by both these investigators suggest that expression of the actin gene in transplanted hearts might be a response to a number of injurious stimuli.

Our analysis has focused more on the β/γ isoforms of actin, which showed the most significant change after transplantation. The α actin, however, also showed a modest increase in transplanted hearts, indicating the activation of this gene along with the β and γ forms. Alpha actins are generally induced by stimuli that lead to cardiac hypertrophy such as stretch and cardiac myocyte-related growth factors. Expression of this isoform of actin is thought to signal regeneration of the mature myocardium [19] and is reasonably to be expected in transplanted hearts, which are likely to have injured myocytes. In our model, the transplanted hearts were rejected due to lack of immunosuppression. Cyclosporin A (CyA) caused immunosuppression in these hearts and would probably have led to a decrease in the α actins, since this CyA has been shown to inhibit cardiac hypertrophy in rats [20]. This could have led to an even greater increase in the ratio of the β/γ to alpha actins.

The implications of the induction of the immediate early genes are not fully understood, though generally considered beneficial [1]. Proteins encoded by these genes may transduce intracellular signals. As the actins are more or less concerned with cell shape and contractility [25], changes in their expression could affect the contractile capacity of the myocytes in the transplanted hearts and their function. Other, more subtle effects of actin expression relating to dysfunction after injury are also known. Translocation of actin filaments to the nuclear membrane is associated with transcriptional activation of genes [7, 24, 32]. Expression of the actin gene in transplanted hearts could thus lead to effects ranging from contractile changes to a cascade of expression of other genes.

As mentioned earlier, the early events after transplantation are increasingly being recognized to influence long-term graft function. For example, one consequence of the early trauma to the transplanted organs is a hyperexpression of MHC class II antigens. In transplanted mouse hearts, we observed a de novo hyperexpression of donor MHC genes in both allo- and isografts [33]. Cell surface expression of the MHC molecules, paralleling their gene expression, is short-lived in the isografts but protracted in the allografts, suggesting that rejection may be mediated by T cell recognition of the allo-MHC on the donor hearts [11]. In their promoter regions, the MHC class II genes and the actin genes share AP-1 binding elements responsive to transcription oncogene c-fos and c-jun [6, 2]. Thus it is possible that repercussion injury sets in motion a cascade of transcription events, starting with the activation of the immediate early genes and progressing to expression of MHC and other immunologically relevant genes in the transplanted organs. A link between the immediate early genes induced by injury and immunologic genes could be the bridge between shock, trauma, and transplantation.

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