Gene program for cardiac cell survival induced by transient ischemia in conscious pigs

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Therapy for ischemic heart disease has been directed traditionally at limiting cell necrosis. We determined by genome profiling whether ischemic myocardium can trigger a genetic program promoting cardiac cell survival, which would be a novel and potentially equally important mechanism of salvage. Although cardiac genomics is usually performed in rodents, we used a swine model of ischemia/reperfusion followed by ventricular dysfunction (stunning), which more closely resembles clinical conditions. Gene expression profiles were compared by subtractive hybridization between ischemic and normal tissue of the same hearts. About one-third (23/74) of the nuclear-encoded genes that were up-regulated in ischemic myocardium participate in survival mechanisms (inhibition of apoptosis, cytoprotection, cell growth, and stimulation of translation). The specificity of this response was confirmed by Northern blot and quantitative PCR. Unexpectedly, this program also included genes not previously described in cardiomyocytes. Up-regulation of survival genes was more profound in subendocardium over subepicardium, reflecting that this response in stunned myocardium was proportional to the severity of the ischemic insult. Thus, in a swine model that recapitulates human heart disease, nonlethal ischemia activates a genomic program of cell survival that relates to the time course of myocardial stunning and differs transmurally in relation to ischemic stress, which induced the stunning. Understanding the genes up-regulated during myocardial stunning, including those not previously described in the heart, and developing strategies that activate this program may open new avenues for therapy in ischemic heart disease.

apoptosis | gene expression | stunning

One of the most important therapeutic targets in the treatment of cardiovascular disease has been the protection of ischemic myocardium from necrosis. This has been a major focus for basic and applied research over the past 30 years. More recently, mechanisms of programmed cardiac cell death (apoptosis) have also been studied extensively. Both necrosis and apoptosis result in the irreversible loss of contractile performance. An unexplored corollary to protection from cell death is the enhancement of cell survival. Our hypothesis is that myocardial ischemia elicits a genomic profile promoting cell survival, which would include the up-regulation of genes involved in prevention of apoptosis, cytoprotection, and cell growth. If a program of cell survival can be stimulated in the ischemic heart, it would represent a novel and important therapeutic strategy in the future.

To address this hypothesis, we examined the genomic profile of ischemic myocardium in a model that is most relevant to clinical conditions, i.e., a swine model of transient ischemia. Although the majority of investigations on myocardial ischemia are conducted in rodent models, major differences exist between rodents and larger mammals (differences in heart rate, action potential, and calcium handling) (1, 2). We reasoned that the best experimental model to elicit a program of cell survival should include a transient episode of ischemia/reperfusion without irreversible damage. This model induces myocardial stunning, which may be one of the most frequently encountered sequelae of ischemia in patients with ischemic heart disease (3). Stunning is the prolonged dysfunction of the ischemic heart that persists after reperfusion despite the normalization of blood flow and that eventually resolves with complete contractile recovery, provided no other ischemic episode intervenes (4, 5). The activation of a program of cell survival would explain both the full reversibility of dysfunction in stunned myocardium and the protection against further ischemia, referred to as preconditioning (6, 7).

Our findings show that transient ischemia followed by prolonged stunning elicits a genomic profile of cell survival, including genes not previously characterized in myocardium. The genomic response parallels the time course of myocardial stunning and differs transmurally, related to the transmural differences in reduction of blood flow during ischemia.

Methods

Animal Model. Female domestic swine (22–25 kg) were sedated with telazol (5 mg/kg, i.m.). General anesthesia was induced by thiopental sodium (5–10 mg/kg, i.v.) and maintained with isoflurane (0.5–1.5 vol%). A left thoracotomy was performed through the fifth intercostal space to expose the heart. A miniature pressure gauge was implanted in the left ventricular cavity to obtain left ventricular pressure and dP/dt. Piezoelectric crystals were implanted in the subendocardium and subepicardium of both the area at risk and the remote area. A hydraulic occluder was implanted around the base of the left anterior descending (LAD) artery. Myocardial blood flow through the LAD was monitored by a Transonics (Ithaca, NY) flow probe. After 3–5 days of recovery, stunning was induced in the conscious animal by inflating the coronary occluder to reduce the blood flow in the LAD by 40–50% from baseline. Reduction of the blood flow was controlled on-line via the flow probe. The coronary stenosis was maintained for 90 min, followed by complete deflation of the occluder and reperfusion. Measurement of regional blood flow by microspheres was performed as described previously (8). All hemodynamics were recorded in the conscious state. Animals were anesthetized with phenobarbital and killed at the end of the 90-min stenosis period, or after 1- and 12-h reperfusion ($n = 5$ in each group). Myocardial samples were taken from both the stunned area (centrally in the LAD territory) and the remote area of the beating heart and were further separated in subendocardial and subepicardial portions. Samples were frozen in liquid nitrogen. Three instrumented pigs, in which

Abbreviations: LAD, left anterior descending; RT-PCR, reverse transcription–PCR; qPCR, quantitative RT-PCR; IAP, inhibitor of apoptosis; PAI, plasminogen activator inhibitor.

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no occlusion was performed, were used as shams. In three additional pigs, cardiomyocytes were isolated from the subendocardial section of the remote and stunned areas after 90-min coronary artery stenosis and 1-h reperfusion, by using techniques described previously (9). These preparations contained more than 95% viable cardiomyocytes.

cDNA Subtractive Hybridization. Total RNA was first extracted (10) from both ischemic and control areas of two hearts submitted to 90-min occlusion and 1-h reperfusion. Messenger RNA was isolated, and 2 μg was used for first-strand cDNA synthesis with random primers. The subtractive hybridization was performed with the PCR-select cDNA subtraction kit (CLONTECH), following the manufacturer's recommendations. After second-strand synthesis, the two cDNA libraries were digested with *Rsa*I. Digestion products of the "tester" library were ligated to a specific adapter (T7 promoter), then hybridized with a 30-fold excess of the "driver" library for subtraction. After hybridization, the remaining products were further amplified by PCR. In the forward subtraction, which determines the genes that are overexpressed in the ischemic sample, the ischemic tissue is the "tester" and the remote sample is the "driver." In the reverse subtraction, the "tester" and the "driver" are switched to determine the genes that are down-regulated in the ischemic sample. PCR-amplified subtracted products were subcloned into the pGEM-Teasy vector (Promega) and transformed into SURE2 cells (Stratagene). The clones were sequenced by standard procedure (ABI-Prizm 377 DNA sequencer, Applied Biosystems). Sequences were queried in public databases to determine the identity of the genes.

Northern Blotting. Fifteen micrograms of total RNA was applied on a 1.2%-agarose denaturing gel and transferred overnight. Probes were derived as isolated restriction fragments from library clones, heat-denatured, and labeled with [α^{32}P]dCTP (Prime-It II kit, Stratagene). Hybridization was performed overnight at 42°C in presence of 50% formamide.

Quantitative Reverse Transcription–PCR (RT-PCR). Quantitative RT-PCR (qPCR) (7700 Prizm, Perkin–Elmer/Applied Biosystems) was performed with specific primers and fluorogenic probes (derived with FAM and TAMRA). For each measurement, the mRNA of interest was reverse-transcribed and subsequently used for two-step qPCR. Internal standards were prepared for each transcript from its PCR-amplified cDNA after ligation of the T7 promoter (Ambion, Austin, TX) (11). Because of variation in sample-to-sample loading, PCR data are reported per number of cyclophilin transcripts measured in each sample.

Nonradioactive *in Situ* Hybridization. Tissue biopsies were taken from remote and stunned myocardium after 90-min occlusion and 1-h reperfusion. Tissue was fixed in 4% paraformaldehyde/PBS, embedded in paraffin, and sectioned at 6-μm intervals. Sections were dewaxed, rehydrated in ethanol, and treated with 0.8% pepsin in 0.2 M HCl (Dako) for 5 min at 37°C, followed by a 5-min rinse in H_2O. Sections were then refixed for 20 min in 4% paraformaldehyde dissolved in PBS. After washing for 5 min in H_2O, sections were acetylated for 10 min in 0.25% acetic anhydride diluted in 0.1 M triethanolamine buffer (pH 8.0), followed by a new wash in H_2O for 5 min. Sections were hybridized overnight at 37°C in a humidified chamber with biotin-labeled oligonucleotide probes diluted in hybridization solution (Dako). The biotinylated probes (synthetized at the New Jersey Medical School Molecular Resource Facility) correspond to the fluorescent probes used for qPCR. After a stringency wash (Dako) for 30 min at 37°C, probe hybridization was detected with streptavidin/alkaline phosphatase, after addition of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a chromogenic substrate (Dako).

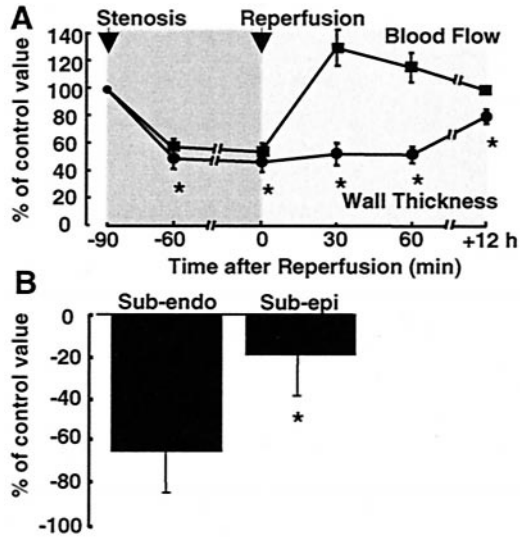

Fig. 1. Time course of functional recovery in stunned myocardium. *A* shows the anterior wall thickness in closed circles and the blood flow through the LAD artery (closed squares), as a percentage of baseline value ($n = 5$ in each group). *B* shows the measurement of blood flow by microspheres in subendocardium and subepicardium of ischemic myocardium at the end of the 90-min ischemia episode (reported as a percentage of baseline value). *, $P < 0.05$ versus baseline value.

Statistical Analysis. Data are expressed as mean ± standard deviation. The number of samples in each experiment is indicated in the figure legends. Statistical analysis was performed with Student's t test. A value of $P < 0.05$ was considered significant.

Results

Functional Characteristics of the Model. Experiments were performed in a swine model of regional low-flow ischemia in the territory of the LAD coronary artery. In basal conditions in conscious swine, the coronary blood flow in the LAD artery was 25 ± 7 ml/min. During the 90-min coronary stenosis, the LAD artery blood flow decreased by 46 ± 6% (Fig. 1*A*). Coronary reperfusion was immediately followed by reactive hyperemia and then a return of flow to baseline values (Fig. 1*A*). As shown in Fig. 1*B*, there was a transmural gradient of blood flow restriction, as measured by microspheres. Whereas the blood flow was reduced by 65 ± 10% in the subendocardium, it was reduced by only 20 ± 17% in the subepicardium (Fig. 1*B*). Therefore, even without considering that wall stress is higher subendocardially, the ischemic stress was significantly greater ($P < 0.001$) in the subendocardium.

The effects of ischemia and reperfusion on wall thickening are shown in Fig. 1*A*. The 90-min coronary stenosis decreased the anterior wall thickness from 3.3 ± 0.9 mm to 1.6 ± 0.4 mm ($P < 0.01$), which corresponds to a 47 ± 7% reduction from baseline. Despite the normalization of blood flow after reperfusion, the contractile function remained depressed ($P < 0.05$) at 1 h (−48 ± 6%) and 12 h (−25 ± 5%), which reflects myocardial stunning. In other experiments, a full functional recovery is typically observed after 48–72 h (not shown). Ischemia did not affect the posterior wall thickening, referred to as the control area. Pathological examination of myocardial samples at 12- and 72-h reperfusion confirmed the absence of necrosis and apoptosis (not shown).

Table 1. Genes of cell survival identified from the subtractive hybridization

Category	Identification	Accession no.
Apoptosis	Inhibitor of Apoptosis	U79142
	Plaminogen Activator Inhibitor-1	Y11347
	Plaminogen Activator Inhibitor-2	Y00630
	EGR-1	X52541
Cytoprotection	Atrial Natriuretic Factor	M13145
	HSP-70	M69100
	HSP-28	AF083246
	HSP-22	AF250138
	α B-crystallin	S45630
	Ferritin heavy chain	D15071
	BAG-family chaperone	AF095193
	5′-AMP Kinase, γ subunit	AK001887
Growth	Cardiac Ankyrin Repeat Protein	X83703
	ATF-3	L19871
	Ribosomal protein S6	J03537
	Ribosomal protein S12	X79417
	Elongation factor-1α	AF013213
	Protein kinase H11	AF133207
	Connective tissue growth factor	AF127918
	Elongation factor 2	M76131
	TACC2	AF095791
	Endonexin II	J03745
	Doc-1	AF006484

Subtractive Hybridization. Stunned and remote samples of two hearts submitted to 90-min stenosis followed by 1-h reperfusion were used for polyA RNA extraction and subtractive hybridization. The clones that emerged from the subtraction were selected randomly for sequencing. A total of 481 clones were successfully isolated and sequenced, including 286 clones from the forward library (genes up-regulated in stunned myocardium) and 195 clones from the reverse library (genes down-regulated in stunned myocardium). Among these 481 clones, 303 (63%) corresponded to nuclear-encoded genes, whereas 178 represented mitochondrial DNA. Of the nuclear-encoded genes, 194 different gene products (64%) were identified. These included 117 products corresponding to known genes (60%), the remaining being undefined and unknown gene products. Among the known genes, 74 (63%) were found to be up-regulated in ischemic tissue, whereas the remaining were down-regulated in stunned myocardium compared with control.

The complete list of known genes that were sequenced from the subtractive hybridization is available as Table 2, which is published as supplemental data on the PNAS web site, www.pnas.org. As shown in Table 1, 31% (23/74) of the genes that were up-regulated in stunned myocardium are involved in different mechanisms of cell survival, including resistance to apoptosis, cytoprotection (''stress response''), and cell growth.

Pattern of Gene Expression Supporting Cell Survival. The genes related to cell survival (antiapoptosis, cytoprotection, and cell growth) were analyzed further by Northern blotting and/or qPCR, comparing stunned and remote areas of five hearts submitted to 90-min occlusion followed by 1-h reperfusion. This time point was selected because it corresponded to the time point at which the samples were taken for the subtractive hybridization. Of the 23 genes in Table 1, 11 were analyzed by Northern blot and/or qPCR and found to be up-regulated in stunned myocardium. Examples of these genes are illustrated in Figs. 2 and 3, emphasizing genes related to resistance to apoptosis.

The transcripts of PAI-1, PAI-2, and EGR-1 were clearly up-regulated in stunned myocardium compared with control (Fig. 2*A*). The signal intensity was measured and reported to the intensity of the 28S rRNA for semiquantitation, showing that EGR-1, PAI-1, and PAI-2 increased more than 10-fold (Fig. 2*B*). Similarly, the expression of inhibitor of apoptosis (IAP) was doubled in stunned myocardium compared with control (Fig. 3). The plasminogen activator inhibitors PAI-1 and -2 are serine proteinase inhibitors with antiapoptotic potential (12–15). The expression of EGR-1 promotes growth and inhibits apoptosis (16–20). The IAP blocks the activation of several caspases (21). Interestingly, we did not find any up-regulation of proapoptotic genes in the library. To test whether other apoptosis-related genes are affected in this model, we specifically measured by qPCR the expression of the proapoptotic genes Bad and Smac/Diablo, as well as the expression of the antiapoptotic gene Bcl-2. At 1-h reperfusion, we did not find any significant difference in the expression of these three genes between stunned and remote areas (not shown).

Other categories of genes supporting cell survival were also up-regulated (Table 1). Most notably, stunning induced a gene program potentially supporting cell growth and cytoprotection. Several genes involved in the stimulation of cell growth and protein translation were up-regulated in stunned myocardium (Table 1). For example, the transcription factor ATF-3, which stimulates cell growth and protein synthesis (22), CARP, a transcription factor involved in cardiac development (23) and cardiac hypertrophy (24), and H11 kinase, a mediator of cell growth and differentiation (25), were all significantly increased. The expression of genes involved in cytoprotection (atrial natriuretic factor, heat-shock proteins HSP-70, HSP-27, HSP-22, and αB-crystallin) was also increased significantly in stunned myocardium.

Time Course of Changes in Gene Expression. qPCR was used to determine whether the time course of changes in the expression of genes related to cell survival replicates the time course of functional recovery in stunned myocardium. A specific quantitative assay was designed for the antiapoptotic genes (*PAI-1*, *PAI-2*, *EGR-1*, and *IAP*). The transcripts of H11 kinase and HSP-70 were measured as markers of cell growth and cytoprotection, respectively. The transcripts were measured in ischemic and remote samples from animals submitted to 90-min stenosis and 0-, 1-, or 12-h reperfusion (Fig. 3). Samples of myocardium from sham-operated animals were also examined, and the gene expression in these samples was found to be similar to that in the remote area of hearts subjected to ischemia and reperfusion, confirming that the remote area was not affected by the protocol.

Whereas the level of the transcripts illustrated in Fig. 3 was already increased at the end of ischemia, the maximal increase in expression was observed at 1-h reperfusion, ranging from 2.5- to 12-fold. Transcript levels returned to normal by 12-h reperfusion, except for *EGR-1* (Fig. 3). It seems therefore that the profile of the changes in gene expression correlate with the profile of functional recovery of the stunned myocardium (Fig. 1), as contractile function has returned to 85% of normal value at 12-h reperfusion, when gene expression tends to normalize.

Transmural Differences in Gene Expression. In each qPCR measurement, the transcripts were measured separately in subepicardial and subendocardial portions of the samples, to test whether the transmural gradient of flow reduction (Fig. 1*B*) induced a similar gradient in gene response. Remarkably, the increased expression of the different genes was consistently higher in the subendocardium over the subepicardium (Fig. 3). These results illustrate for the first time, to our knowledge, that the myocardial gene response in stunned myocardium in large mammals follows a transmural gradient, which corresponds to the transmural gradient of blood flow reduction during the ischemic insult.

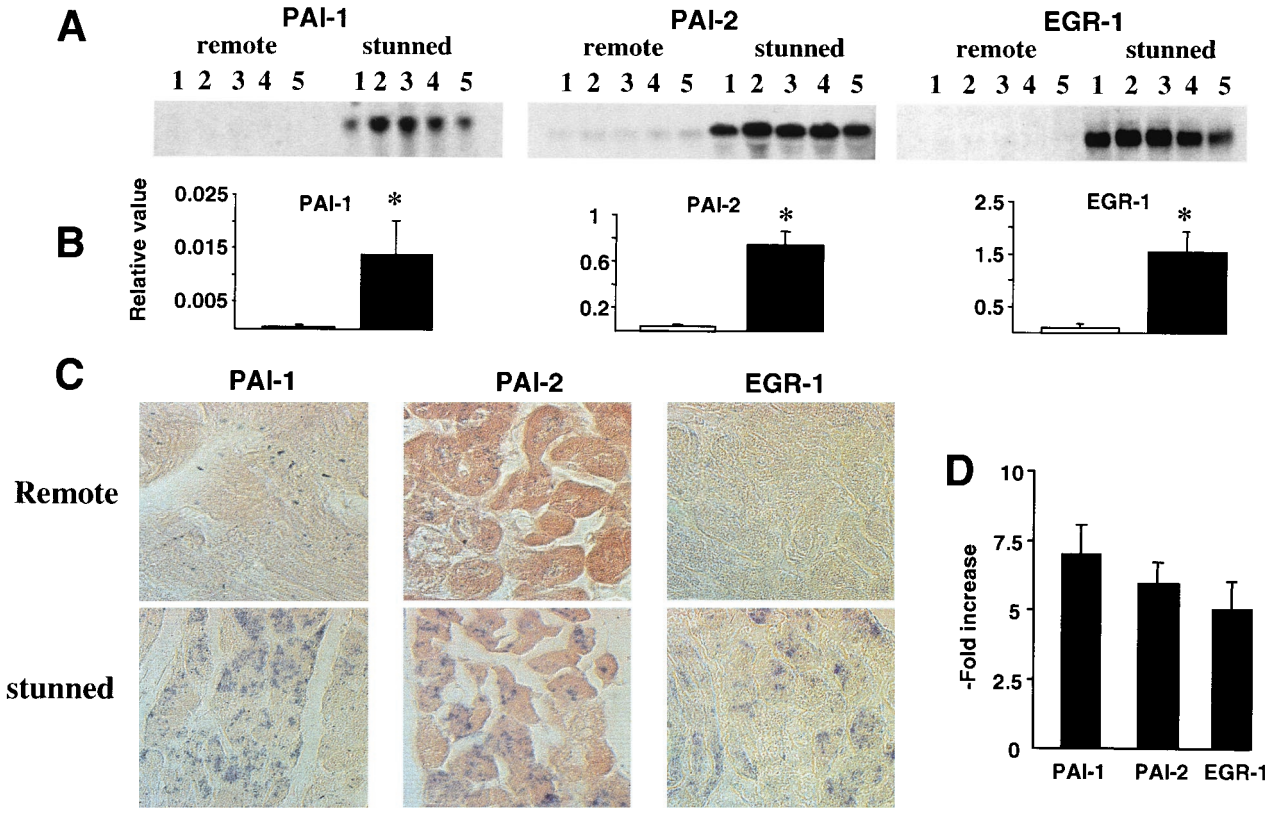

Fig. 2. Changes in gene expression analyzed by Northern blotting and *in situ* hybridization. (*A*) Northern blot was performed on samples of the remote and stunned area from five different hearts at 1-h reperfusion. Transcripts coding for PAI-1, PAI-2, and EGR-1 are illustrated. (*B*) Differences after 18S rRNA normalization between remote myocardium (open bars) and stunned myocardium (closed bars). *, $P < 0.05$ versus remote. (*C*) Tissue distribution by *in situ* hybridization. A clear induction was observed in cardiomyocytes from the ischemic area (stunned). (*D*) Changes in gene expression quantitated in isolated cardiomyocytes, prepared from the remote and stunned area of three different hearts submitted to 90-min ischemia and 1-h reperfusion. The graph illustrates the *n*-fold increase of PAI-1, PAI-2, and EGR-1 transcripts in stunned over normal myocytes.

Tissue Distribution of Transcripts. It remains so far unknown whether transcripts such as *PAI-1*, *PAI-2*, or *EGR-1* are expressed in the cardiomyocytes, in addition to other cardiac tissues. We relied on *in situ* hybridization experiments to answer that question. Although no clear signal could be detected in normal myocardium, a clear induction of these transcripts was observed in the cardiomyocytes from stunned myocardium (Fig. 2*C*). To quantitate these changes, we measured by qPCR the expression of these genes in cardiomyocytes isolated from three different hearts using the same protocol, i.e., an episode of 90-min coronary stenosis followed by 1-h reperfusion. By comparing the stunned and remote areas, we confirmed that the changes in expression of *PAI-1*, *PAI-2*, and *EGR-1* occurred in cardiomyocytes (Fig. 2*D*). Similarly, the increase in stunned over normal myocytes was 4.3 ± 0.2-fold for *H11 kinase*, 3.0 ± 0.5-fold for *HSP-70*, and 1.8 ± 0.3-fold for *IAP* ($P < 0.05$).

Discussion

It is well recognized that myocardial ischemia leads to cell death, whether by necrosis or apoptosis, and that survival of postischemic myocardium depends on factors that limit necrosis and/or apoptosis. The results of the present investigation propose a new concept, i.e., that ischemia followed by reperfusion induces a gene program of cell survival. This means myocardial ischemia and reperfusion elicit antagonistic forces, directing the myocyte toward either death or survival. It will be important to understand factors that may tilt this balance, which potentially will provide novel therapeutic directions. An additional unique feature is that the study was conducted in a large mammalian

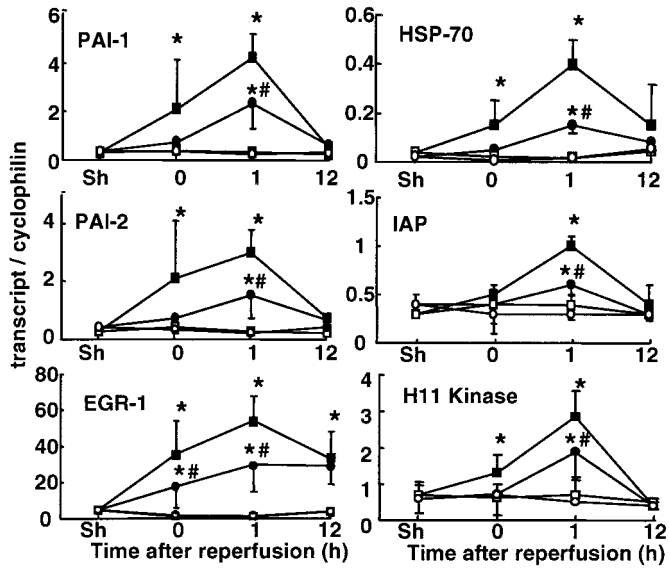

Fig. 3. Time course of changes in gene expression analyzed by qPCR. Transcripts were quantitatively measured in samples of the remote (open symbols) and stunned area (closed symbols) from hearts submitted to 90-min occlusion followed by no reperfusion or 1- or 12-h reperfusion ($n = 5$ in each group). The subendocardial (squares) and subepicardial areas (circles) were measured separately and compared with sham-operated animals (Sh). *, $P < 0.05$ versus corresponding value in remote myocardium. #, $P < 0.05$ versus corresponding value in subendocardium.

model of coronary stenosis and reperfusion, because the majority of our knowledge of functional genomics is based on experiments conducted in rodent models.

Functional Genomics in Large Mammals. Schaper's laboratory provided pioneering studies on alterations in gene expression in swine models of ischemia/reperfusion (20, 26). These studies examined specifically the time course of changes in genes encoding protooncogenes and calcium-handling proteins (20, 26). In contrast, we applied recently developed techniques of gene screening to determine the global change in gene expression in response to transient ischemia, rather than selecting specific targets. Although several recent studies have investigated the genome profile of the hypertrophied and infarcted myocardium in rodents (27–29), our study is the first to apply this approach in large mammals but also is the first, to our knowledge, to show a program of cell survival in myocardial ischemia/reperfusion (myocardial stunning).

There are major species differences between rodents and large mammals, both at the level of fundamental regulation of cardiac contraction and in the mechanisms mediating myocardial stunning (30, 31). The lack of collaterals in the swine heart makes this model particularly relevant to clinical conditions. Other advantages of the model include the possibility of inducing regional ischemia, the accurate control of flow reduction, and the clear distinction between ischemic and remote areas. The use of chronically instrumented conscious animals is closer to physiological conditions and avoids the complicating factors of recent anesthesia and surgery. Our results show that the remote area is affected neither at physiological nor at transcriptional levels by the protocol. Another unique feature of the preparation is that it allows a separate analysis of subendocardium and subepicardium, which has not been examined before.

We show that the alteration of gene expression induced by ischemia is systematically more profound in subendocardium than subepicardium (Fig. 3), which corresponds to a more severe reduction of blood flow in the subendocardium (Fig. 1*B*), in combination with a higher subendocardial wall stress. Therefore, the transmural gradient of physiological stress is translated into a gradient at the level of gene expression. Thus, the gene response appears finely tuned by the amplitude of the ischemic stress, contrary to an "all-or-nothing" response. The absence of changes in the remote area also reinforces the specificity of this response.

The question that arises is what is the stimulus for this gene signature, and how does it relate to stunning? That the stimulus starts during ischemia suggests that it is ischemia rather than stunning that functions as a trigger. This is reinforced by the transmural gradient of the gene response, correlating the intensity of gene response during stunning with the intensity of insult during ischemia. However, it must be emphasized that the peak changes in gene expression occur early during reperfusion, which could affect the later phase of recovery of stunned myocardium or even potentially could play a role in preconditioning (32) or myocardial hibernation.

Importance of Genomic Changes. Our results demonstrate that nonlethal ischemia is accompanied by the expression of a genetic program of cell survival that potentially counteracts apoptosis. Although we focused mainly on the antiapoptotic genes, the global program of cell survival shown in Table 1 includes 23 products (31% of the up-regulated genes) involved in antiapoptosis, cytoprotection, and cell growth. In addition, we observed an up-regulation of genes involved in sarcomeric protein synthesis and metabolism, which tangentially may also sustain cell survival (not shown).

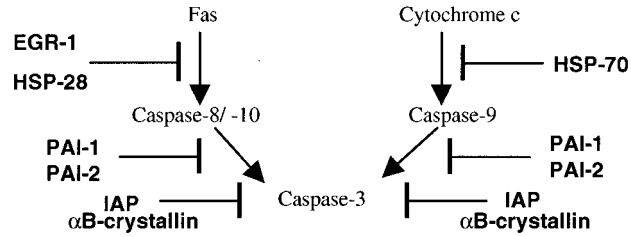

Fig. 4. Action of the antiapoptotic genes detected in the subtracted library. The figure illustrates the two potential pathways of apoptosis activated by ischemia and the site of action of the different antiapoptotic products identified in the library.

Antiapoptotic Response. Transcripts of four potent antiapoptotic genes were increased as much as 12-fold (Figs. 2 and 3). The IAPs inhibit the tumor necrosis factor α-activated apoptosis pathway (33, 34) and also directly inhibit caspase-3 and -7 (21), which identifies IAPs as the most potent inhibitors of cell death described to date (Fig. 4). The plasminogen activator inhibitors PAI-1 and -2 belong to the serpin family of serine proteinase inhibitors, which has an antiapoptotic potential (12–15). Because serpins inhibit caspase activation by serine proteinases (35), they may play a central role in the control of apoptosis. The expression of EGR-1 promotes growth and inhibits apoptosis in different cell types (16–18), including the heart (19, 20). Its antiapoptotic effect includes the down-regulation of the Fas/Apo-1 promoter (36) and the transcriptional stimulation of PAI-1 (37). As mentioned above, we did not find any proapoptotic transcript to be up-regulated in the library. The quantitative measurement of two specific proapoptotic genes (Bad and Smac/Diablo) did not reveal any difference between stunned and normal myocardium. We have therefore no reason to suspect that, in response to the well-demonstrated activation of survival mechanisms, there could be an activation of proapoptotic pathways in our model. However, it remains possible that a more aggressive episode of ischemia can tilt the balance in the other direction.

Cytoprotection. Heat-shock proteins prevent the denaturation of various intracellular proteins (38, 39) and, in that respect, they also participate in cell survival. Most importantly, heat-shock proteins are directly involved in antiapoptotic mechanisms. HSP-70 inhibits the activation of caspase-9 by blocking the association of procaspase-9 to Apaf-1 (40, 41) and prevents the activation of the JNK pathway(42). Small heat-shock proteins, such as HSP-27, block the Fas/FasL pathway (43), whereas αB-crystallin inhibits the maturation of caspase-3 (44). Therefore, these genes increase the list of antiapoptotic products that are up-regulated in ischemic heart. These products act synergistically at different checkpoints of the apoptotic cascade and represent a coordinated mechanism to promote cell survival (Fig. 4).

Growth Response. Antiapoptotic mechanisms are linked to cell growth. This association also applies to our model, although there is no hypertrophy in stunned myocardium. However, it remains possible that repetitive stunning or chronic hibernation may ultimately induce cardiac hypertrophy. Several factors involved in the stimulation of protein translation are up-regulated in stunned myocardium (Table 1). The transcription factor ATF-3 stimulates cell growth and protein synthesis (22). CARP is a transcription factor involved in cardiac development and reexpressed in the hypertrophied heart (24). The expression of H11 kinase is associated with cell growth and differentiation (25). Together with the antiapoptotic genes, this growth program

also represents a mechanism to sustain cell homeostasis and survival in stunned myocardium.

The subtractive hybridization is unbiased by the function of the genes. We therefore did not "select" the survival and antiapoptotic genes, but these are the genes that naturally came out of the library. As a discovery tool, the subtraction is not a quantitative method; we needed to validate the results by alternative techniques (Northern blot, qPCR, *in situ* hybridization, and isolated cardiomyocytes). The subtraction method is limited in that it does not present the entire set of genes that are potentially affected. However, this technique did provide sufficient information to formulate a new concept, i.e., the activation of endogenous mechanisms of cardiac cell survival during ischemia/reperfusion. In the future, the development of DNA chips for the pig could expand on this new concept and delineate all of the genes that are, or are not, regulated by nonlethal ischemia.

Up-Regulation in Stunned Myocardium of Genes Not Previously Described in Cardiac Myocytes. An additional important finding of the current investigation that was facilitated by the subtractive hybridization approach is the observation that several of the genes that were found to be up-regulated in stunned myocardium have not previously been described in cardiac tissue. It is just this type of approach that could open new avenues to understand myocardial disease processes in terms of mechanisms not currently considered. Examples of these genes are PAI-1 and -2, which have been described in endothelial cells and tumors (12, 15, 45). Because one could argue that these genes were up-regulated in the heart, but not in myocytes, e.g., vessels, fibroblasts, macrophage, we needed to confirm that the up-regulation was specific to myocytes. This was accomplished qualitatively by *in situ* hybridization and quantitatively by measuring the expression of these genes in myocytes isolated from the stunned and remote areas. These experiments confirmed the specificity of the gene response to cardiac myocytes.

Therapeutic Perspective. The chronic evolution of ischemic heart disease generally involves a progressive loss of myofilaments (46) and cardiomyocytes (47). Promoting a genomic program for cell survival, including antiapoptotic genes, cytoprotective genes, and genes involved in cell growth, protein synthesis, and sarcomere synthesis, may counteract this trend. Our study clearly shows that such mechanisms are at work in a model of reversible ischemia, which is encountered frequently in patients with coronary artery disease. This genomic program may be responsible in part for the reversibility of nonlethal ischemia in stunned myocardium and may be involved in other syndromes characterized by cell protection, e.g., myocardial hibernation and preconditioning. Reproducing or amplifying the genomic profile described in the current investigation, as well as understanding the role of genes not previously described in cardiac myocytes, may open new avenues to treat ischemic heart disease.

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