

# Correspondence

## Chromosome 6 Abnormalities Correlated with Thymoma Progression

### To the Editor-in-Chief:

Using comparative genomic hybridization (CGH) and microsatellite analysis, Inoue et al<sup>1</sup> have characterized some of the common genetic abnormalities found in thymomas.<sup>2-3</sup> The most frequent genetic abnormality detected was loss of genetic material or LOH on the long arm of chromosome 6.

The advent of high-density oligonucleotide microarray technology, with its capacity to simultaneously monitor thousands of genes, also provides a unique opportunity for high-throughput genetic analysis of a tumor. We have examined and reported differential gene expression in patients with invasive/non-invasive thymoma by means of the Affymetrix Hum95000 array (Santa Clara, CA) Biochip (microarray) method.<sup>4</sup> Although we have used the D-chip analysis method in the previous paper cited above,<sup>4</sup> we have now changed the analysis method to Gene Spring analysis (Silicon Genetics Co., Redwood City, CA) and found that several genes at chromosome 6 overexpressed in invasive thymoma (Table 1). In search of genes involved in the progression of thymoma, we compared gene expression between advanced thymoma (two stage IVa B3 cases) and early thymoma (one stage I A and one stage II B3 case) samples.<sup>4</sup> We should mention that the comparative differential gene expression analysis of advanced stage thymoma *versus* early stage thymoma revealed that four genes had significantly altered levels of expression by twofold or greater at 6q21-24 lesions.

A glycosylphosphatidyl-inositol (GPI)-anchored glycoprotein (GPI-80) is highly homologous with vanin-1, a recently reported GPI-anchored protein that is expressed

on perivascular thymic stromal cells and is involved in thymus homing in mice.<sup>5</sup> The finding that both GPI-80 and vanin-1 are 40% homologous with human biotinidase suggests that the existence of a biotinidase superfamily of molecules that may be involved in the regulation of leukocyte trafficking.<sup>5</sup> Hematopoietic tumors in both humans and mice frequently up-regulate expression of the c-myc gene.<sup>6</sup> DNA microsatellite instability for c-myc was reported in colon polyps and adenomas.<sup>7</sup> Human cutaneous fatty acid-binding protein gene is capable of inducing the metastatic phenotype when overexpressed in non-metastatic rat Rama 37 cells.<sup>8</sup> Northern and slot blot analysis revealed that expression of the endogenous vascular endothelial growth factor (VEGF) gene was increased in cutaneous fatty acid-binding protein gene-transfected cells.<sup>8</sup> Brain fatty acid-binding protein is also expressed in a subset of human malignant glioma cells.<sup>9</sup> Finally, the membrane-spanning connexin proteins form microscopic intercellular channels that directly connect the cytoplasm of adjacent cells and as such have been implicated in maintenance of tissue homeostasis. They are considered to act as tumor suppressors since their function or expression is frequently aberrant in tumor cells. The mutational alterations of connexin 43 are involved in advanced stages of progression of human colon cancers.<sup>10</sup>

In our cDNA microarray analysis, we identified several differentially expressed genes at chromosome 6, of which the potential roles in tumor progression have been described previously. However, we could not say whether those overexpressed genes were mutated or not from our analysis. Inoue et al<sup>1</sup> determined that chromosome 6 is a target of frequent chromosomal aberrations in thymoma and suggested the presence of several putative tumor suppressor genes on chromosome 6 that might contribute to the pathogenesis of thymoma. Further MSI or mutation search for these genes in thymoma are

**Table 1.** Representative List of Differentially Expressed Genes at Chromosome 6 between Invasive and Noninvasive Thymoma

Clone ID	GB	Description	Fold change	Map
34498_at	D899974	glycosylphosphatidyl inositol-anchored protein (GPI-80)	233.35	q23-24
39317_at	D86324	CMP-N-acetylneuramic acid hydroxylase	7.495878	p22-23
1476_s_at	U22376	c-myc	6.480358	q22-23
31850_at	M90656	gamma-glutamylcystein synthetase	6.405943	p12
39331_at	X79535	beta tubulin	4.879784	p21.3
37561_at	AL031778	nuclear transcription factor Y	3.245321	p21.3
1261_i_at	M16594	glutathione S-transferase Ha subunit 2	3.153584	p12.2
38052_at	M14539	factor XIII	2.995713	p25.3-24
35185_at	AJ002962	fatty acid binding protein 7	2.931442	q22-23
36773_f_at	M81141	MHC class II HLA-DQ beta	2.797017	p21.3
32531_at	X52947	connexin 43	2.71037	q21-23.2

GB, GenBank number; CMP, cytidine monophospho.

warranted to determine whether the relation with tumor progression of thymoma.

We believe that this *in vivo* functional genomic approach not only provides an evolving opportunity to rapidly and directly monitor *in vivo* gene expression in human thymoma, but also promises to provide novel insights into fundamental cancer biology. Furthermore, the application of this approach to clinical thymoma specimens may provide a key step to rapid advances in thymoma prevention, detection, diagnosis, and therapeutics.

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## Author's Reply:

Sasaki et al<sup>1</sup> have undertaken another step in elucidating which of the plethora of genetic aberrations occurring in thymoma are important in the progression of this disease from early to advanced stages. They examined gene expression patterns of several early and advanced thymomas

looking for differences between those two groups. They came out with a list of genes showing different expression levels. However, their results are speculative at best.

The number of cases investigated (as referred to in the above letter) is completely insufficient. To draw conclusions based on the results obtained on four (moreover, heterogeneous) cases does not allow any meaningful statistical analysis. The low number of cases actually precludes any use of statistics. These results, based on the analysis of four cases, seems to belong to the realm of random error. A somewhat different picture emerges looking at their recent publication.<sup>1</sup> Here, they focused on glycosylphosphatidyl-inositol (GPI)-anchored glycoprotein (GPI-80) and analyzed its levels in the tumor, thymoma, and in peripheral blood. While the GPI-80 mRNA results for thymoma show huge variation, GPI-80 protein serum levels are more consistent. However, I have doubts about the relevance of the data for the clinician in the real life (the test would have a terrible specificity) given the considerable overlap in values not only between different thymoma stages but also between patients with thymoma of any stage, myasthenia gravis, or normal controls.

The above study shows how important it is to use proper statistical methods when analyzing microarray results. Do not pick a reason to prove retrospectively a favorite hypothesis. A much better way how to find meaningful differences between early and late stage thymomas is to look at differences between signaling pathway activation patterns. Only then it will be possible to elucidate the pathway of thymoma development, the succession of the individual aberrations, and their contribution to pathogenesis. That is what we owe to our patients.

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## *VEGF Protein in Human Ischemic Skeletal Muscle*

### To the Editor-in-Chief:

We contact you in regard to the recent publication in your journal of Rissanen et al<sup>1</sup> on the apparent up-regulation of VEGF protein in regenerating fibers in human ischemic skeletal muscle.

Some of the conclusions drawn by the authors appear not to be well founded. Most importantly, we think that the authors have to show critical antibody controls for the VEGF staining. This is essential to exclude the possibility

of non-VEGF-specific staining of regenerating fibers in ischemic muscles.

Another main concern relates to the immunohistochemical data. We can demonstrate with a similar detection system that inclusion of any polyclonal first antibody from rabbit in the immunostaining procedure will cause staining of the cytoplasm of "certain" fibers in regenerating but not normal and atrophied (rat) soleus muscle. We have detected in 14 days regenerating muscle exactly the same pattern of immunoreactive fibers as in the study by Rissanen et al<sup>1</sup> when we used polyclonal VEGF anti-serum or normal rabbit serum (but not PBS alone) as first antibody. According to morphological criteria, the "polyclonal first antiserum" positive fibers in this experiment were found to represent small (regenerating) fibers. A representative example of this experiment is shown in Figure 1. As far as we know, there is no clear cut molecular explanation for this phenomenon but we guess that the muscle regeneration-specific fiber staining may reflect accumulation of IgG, IgG-binding protein (of the complement system) in damaged fibers. A representative example of this experiment is shown in Figure 1.

Unfortunately, the immunoreaction control in Figure 2 of Rissanen et al's paper<sup>1</sup> was done with omission of the first antibody. The omission of first antibody in our control experiments abolished the distinct staining of regenerating fibers seen with addition of normal rabbit serum. The *in situ* data shown in Figure 1 of their paper also does not provide the information necessary to exclude potential VEGF-unspecific fiber staining has occurred as they do not derive from consecutive sections to the VEGF protein data. If a similar immune-reaction occurs in regenerating human muscle, the immunoreactive fibers in the ischemic human legs may arise in consequence of inclusion of a polyclonal first antibody, and not due to its specificity for VEGF, in the immunostaining procedure. This may explain Rissanen's<sup>1</sup> conclusions of VEGF's association with regenerating fibers and macrophages.

To judge the validity of the conclusions of VEGF stain presented in Figure 1 of the Rissanen paper it is therefore

important to demonstrate that no fiber-internal staining is observed with normal serum on sections of ischemic muscle consecutive to the ones stained for VEGF.

As a secondary point, some concerns arise due to the lack of quantitative data on the association of VEGF with regenerating and atrophic fibers as well as capillaries. From the description of the authors it is not clear if VEGF could be found in all atrophic and regenerated fibers or if other possible relations as for example to fiber-types existed. Also, the authors do not state how the counting of capillaries were performed, nor do they state the different frequencies in capillary number in VEGF-positive and -negative fibers and its statistical significance. However, they put forward in their conclusions that the amount of capillaries was increased around the atrophic muscle fibers. In this manner the authors do not acknowledge the fact that a clear description of how this measure was done (capillary density, capillary per fiber, fiber per capillary) is of major importance to understanding the vascular adaptations.<sup>2</sup> Moreover, to us it is not clear if the strongly VEGF positive lumen in the capillary marker CD31 positive structures reflect thrombus formation in blood vessels and if these would contain VEGF (compare Figure 1, e and f). In view of the practical implications we felt it to be necessary to comment on this issue. We would very much appreciate to hear other opinions on these issues.

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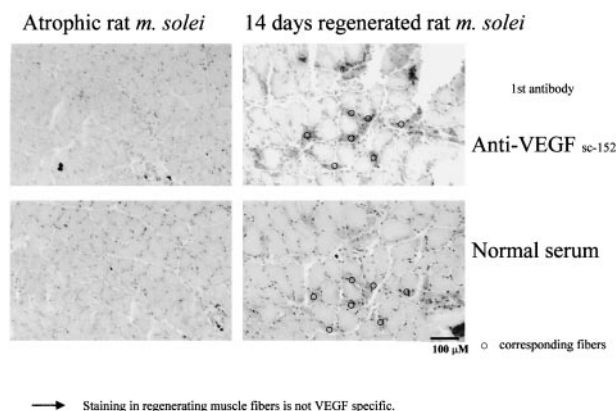
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## Authors' Reply:

We thank Drs. Gustafsson and Flück for their interest in our study. They express concerns about the specificity of VEGF immunostainings done with rabbit polyclonal antiserum on rat skeletal muscle. However, it is difficult to see how these comments relate to our paper since we have used neither rabbit polyclonal antibodies nor rat tissues.<sup>1</sup> All VEGF immunostainings were done on human and rabbit tissues with a mouse monoclonal antibody (Santa Cruz, Clone cs-7269). Also, unlike Drs. Gustafsson and Flück mention in their letter, all immunostainings were



**Figure 1.** Photographs showing corresponding fields of immunostained sections from atrophic (**left**) and regenerating (**right**) rat soleus muscle. Consecutive cryosections (12 μm) were dried, fixed in 4% paraformaldehyde and blocked in 1% casein. Subsequently, sections were incubated with rabbit VEGF antibody sc-152 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; **top**) or normal rabbit serum (**bottom**) and color was developed with diaminobenzidine. Nuclei were counterstained with hematoxylin.

controlled by class- and species-matched immunoglobulins in addition to controls where the primary antibody was omitted. All these points are clearly written in the Methods section. In addition, normal horse serum that was used to block possible unspecific staining showed no staining. VEGF immunostainings were also confirmed by *in situ* hybridizations, which unequivocally demonstrate synthesis of VEGF mRNA in atrophic and regenerating myocytes. It is surprising that Drs. Gustafsson and Flück do not recognize the importance of these findings.

Our results are in agreement with VEGF expression in rat myocardial infarction where the acute widespread VEGF expression was later replaced by VEGF expression restricted to the regions bordering to the infarcted area.<sup>2</sup> Also, in a similar pattern with VEGF expression, regenerating but not normal skeletal myocytes have been previously found to express hepatocyte growth factor<sup>3</sup> and insulin-like growth factors<sup>4</sup> both of which can up-regulate VEGF expression.

Drs. Gustafsson and Flück do not provide any clear explanation for their own findings, which are shown in Figure 1 of their letter, although they speculate possible accumulation of IgG in damaged fibers. In our lab where we have over 20 years' experience in immunostainings and elsewhere it is well known that immunoglobulins can bind to cytoplasm of dying or damaged cells.<sup>5</sup> For that reason, special care was taken to include adequate controls for the immunostainings by Rissanen et al<sup>1</sup> Also, the very specific localized immunostaining for VEGF shown in Figure 4i of our paper<sup>1</sup> is difficult to discount based on the points raised by Drs. Gustafsson and Flück.

Regarding the comment about the lack of quantitative data, it is generally accepted that it is very difficult to provide quantitative immunohistological data. We used a semi-quantitative grading system for VEGF expression that is applicable to this type of studies. The statements on the increased vasculature in VEGF-positive areas in our paper are qualitative and based on consecutive CD31 and VEGF immunostainings. It is also noteworthy that capillary density and capillary/myocyte ratio may not always be the best measures for VEGF-driven angiogenesis in skeletal muscle.<sup>6,7</sup>

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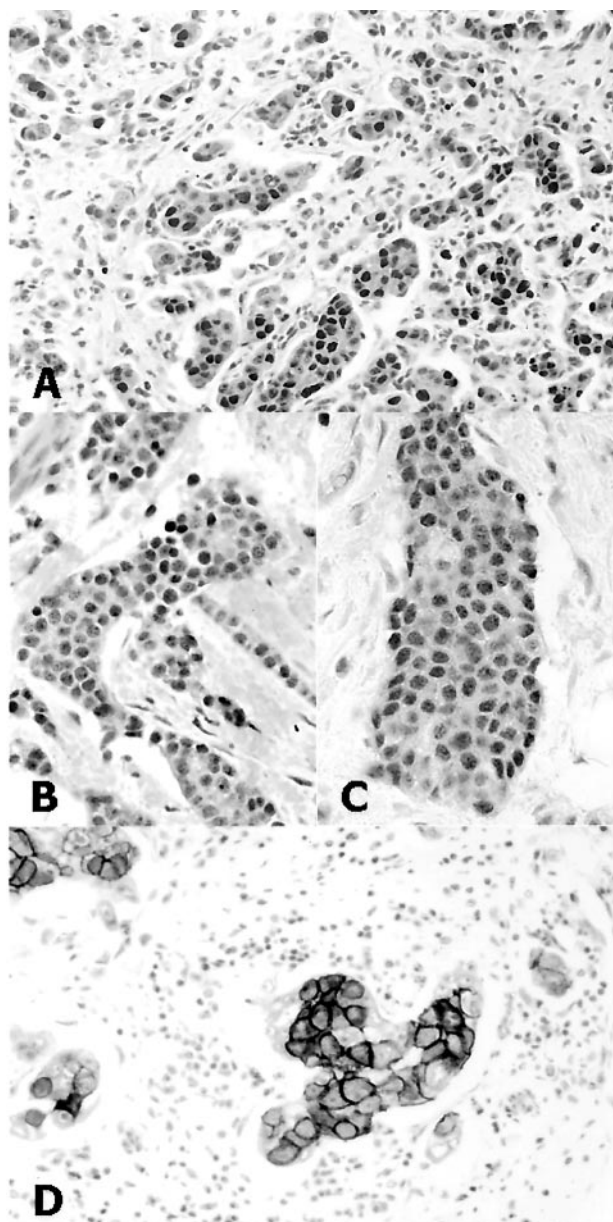
## *What's Cooking? Detection of Important Biomarkers in HOPE-Fixed, Paraffin-Embedded Tissues Eliminates the Need for Antigen Retrieval*

### To the Editor-in-Chief:

Recently, the immunohistochemical assessment of biological parameters has become an integral part of both investigative as well as diagnostic pathology. Cell proliferation is often measured in tumors and the determination of hormone receptors is mandatory in the reporting of breast cancer.<sup>1–3</sup> Finally, there are FDA-approved immunohistochemical assays which are decisive for adjuvant therapy, eg, the HercepTest. To our knowledge, however, there are no assay systems available applicable to routinely formalin-fixed and paraffin-embedded material without antigen retrieval, which in most cases means, in effect, cooking of the specimens. The mechanism underlying this is poorly understood. Moreover, the procedure often leads to a loss in the morphological quality and the repeated heating and cooling are very difficult to standardize.<sup>4</sup> Recently it has been described that the HOPE (Hepes-glutamic acid buffer mediated Organic solvent Protection Effect, German patent DE 10021390 C2) fixation and subsequent direct paraffin-embedding results in a morphological preservation of human soft tissues which is well comparable with routinely formalin-fixed and paraffin-embedded specimens.<sup>5</sup> As part of an investigation aimed at elucidating the possibilities and limitations of HOPE-fixation, we analyzed the immunohistochemical assessment of cell proliferation using the common MIB-1 antibody.<sup>2</sup> From the group of important and relevant hormone receptors we studied the detectability of estrogen- and progesterone-receptors<sup>6</sup> and of FDA-approved assays we applied the HercepTest.<sup>7</sup> The assessment of all these important biomarkers in paraffin sections requires cooking when formalin fixation is used.

HOPE-solution was obtained from Dr. J. Olert (contact: olert@kinderpatho.klik.uni-mainz.de). Formalin-fixed and





**Figure 1.** Immunohistochemistry applying HOPE-technique without antigen retrieval on breast cancer sections. **A:** Ki-67 (MIB-1,  $\times 200$ ). **B:** Estrogen-receptor (1D5,  $\times 400$ ). **C:** Progesterone-receptor (PgR 636,  $\times 400$ ). **D:** c-erb B2/neu (HercepTest,  $\times 200$ ).

correspondingly HOPE-fixed, paraffin-embedded tissues of mammary carcinoma specimens were cut into 4- $\mu$ m sections. Sections were deparaffinized and the primary antibodies were applied in moist chambers.<sup>5</sup> Endogenous peroxidase was inactivated by 3%  $H_2O_2$  for 10 minutes at ambient temperature. Negative controls were included from every specimen. Formalin-fixed samples from the same tumors as the HOPE-fixed were included, conventionally deparaffinized and treated or not treated with the appropriate antigen retrieval techniques. Specimens were blocked for non-specific binding by incubation in normal, heat-inactivated pig serum (1:30 in Tris-buffered saline) for 10 minutes at ambient temperature. The applied dilutions and incubation times of the primary

antibodies were: (a) 1 hour at ambient temperature: MIB-1 (1  $\mu$ g/ml for formalin-fixed sections, 333 ng/ml for HOPE-fixed sections); and (b) overnight at 4°C: estrogen-receptor (Dako, 1D5, 1:75 dil. for formalin-fixed sections; 1:225 dil for HOPE-fixed sections), progesterone-receptor (Dako, PgR 636, 1:100 dil. for formalin-fixed sections; 1:300 dil. for HOPE-fixed sections)

Detection of HOPE-treated material was achieved by LSAB2 (Dako) diluted 1:3 for MIB-1, estrogen- and progesterone-receptor, or EnVision (Dako) diluted 1:3 for the HercepTest. In the case of formalin-fixed specimens, detection was achieved by LSAB2 or Envision according to the manufacturer's recommendations. Sections treated with LSAB were developed using 3-amino-9-ethylcarbazole/ $H_2O_2$ , the HercepTest was developed with diaminobenzidine/ $H_2O_2$  according to the protocol of the manufacturer. Subsequently all specimens were counterstained with hemalum and mounted.

The formalin-fixed specimen without antigen retrieval remained negative staining (not shown). As demonstrated in Figure 1 we achieved very good immunostaining for all important biomarkers investigated in HOPE-fixed paraffin sections without antigen retrieval. It is important to note that in terms of quantity and intensity of positively stained cells there was almost no difference between the established protocols for formalin-fixed materials using antigen retrieval and the HOPE-protocol without the latter, with the HOPE-fixed specimens requiring only one-third of the primary antibodies as well as one-third of the respective detection system.

Thus HOPE-fixation, due to the proved excellent preservation of antigens, the highly standardized tissue-sampling, and the well preserved morphology meets the new and increasing demands in the expanding field of molecular diagnostics, and we propose that it should be considered in this respect as an improved alternative fixation method in routine immunohistology.<sup>8-10</sup> Appropriate large scale studies are underway.

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## Unusual Apoptosis in Experimental Cardiac Rejection

### To the Editor-in-Chief:

Being interested in the histopathology of cardiac rejection,<sup>1</sup> I was pleased to read the constructive article by Miura and co-workers<sup>2</sup> about the transplantation of hearts from A/J (H-2<sup>a</sup>) mice to major histocompatibility complex mismatched recipients divided further into two groups: wild-type (WT) C57Bl (H-2<sup>b</sup>) and interferon (IFN)- $\gamma$  deficient C57Bl/IFN- $\gamma$ <sup>-/-</sup>. The allografts were rejected in about 8.5 days in WT recipients by a severe cellular acute rejection mediated by CD8<sup>+</sup> T cells and in about 6 days in IFN- $\gamma$  deficient hosts by a rejection manifesting intense infiltration by neutrophils. The analysis of Figure 2 of Miura et al's<sup>2</sup> article, visualizing basic histopathology of the rejecting cardiac allografts about 1 day before the end of rejection, is the subject of this letter.

In substance, I agree with the authors as far as Figure 2, a and c, are concerned. There are still some well-preserved cardiomyocyte nuclei in Figure 2c. I mention this fact because absolute magnification numbers accompanying published microphotographs are often imprecise due to printing procedures. I will compare, therefore, the approximate diameter of cardiomyocyte nuclei (4  $\mu$ m) in Figure 2c ( $\times 200$ ) with one structure in Figure 2d ( $\times 200$ ) to determine its dimensions.

I also agree with the authors regarding the neutrophil infiltration in Figure 2, b and d. However, I do not see "diffuse intense intragraft thrombosis" in Figure 2, b and d. It is quite possible that small microthromboses exist in the heart in question, but I have not noticed any identifi-

able thrombus in this figure. What factual information supports the "thrombosis" statement?

I have been struck most by a formation of myocardial defects which are well visible in Figure 2b. One of them is seen in detail in Figure 2d. These defects may be mistaken for vessels at first sight. However, they manifest the features which are incompatible with vascular origin: they do not possess vessel walls; they often fuse gradually with the surrounding myocardium (Figure 2d, upper right quarter); they contain fragments of cardiomyocyte cytoplasm (Figure 2d, lower left center, at the "7 o'clock" position); and they comprise cells with nuclei similar to the nuclei of cardiomyocytes and surrounded by a narrow rim of cytoplasm with cardiomyocyte tinctorial properties (Figure 2d). Some of these nuclei are practically "naked."<sup>3</sup>

What pathological process has created such myocardial defects within five days after transplantation? Is it the necrosis mediated by neutrophils and mentioned by the authors?<sup>2</sup> Myocardial necrosis is phagocytized by macrophages. This is a process lasting days, weeks, and months which is followed immediately by healing reaction and scarring. Consequently, necrosis would not have formed myocardial defects filled with interstitial fluid (Figure 2, b and d), and one must look for another explanation for their appearance.

To do so, let's pay attention to the "severe disseminated hemorrhagic necrosis," which is the third important pathological process mentioned by the authors in Figure 2, b and d.<sup>2</sup> In cardiac pathology, the term "hemorrhagic necrosis" is used to designate necrotic myocardium with blood extravasated into the interstitial space. It is often described in hemorrhagic infarcts, hyperacute rejection, and other pathological processes. Its concept suffers, however, from numerous shortcomings. For example, interstitial spaces between cardiomyocytes are extremely narrow (from 0.2  $\mu$ m to a few  $\mu$ m) and blood pressure is not high enough to dislodge cardiomyocytes from their original position.<sup>4</sup> It is difficult, therefore, to account for large accumulations in red cells in the narrow interstitial spaces. Most often, alleged extravasated blood contains only erythrocytes and lacks an adequate amount of fibrin. Furthermore, it is a process which is accompanied by an unaccounted loss of cardiomyocytes.<sup>5</sup> To explain these contradictions, one current theory proposes that alleged red cells present in "hemorrhagic necrosis" are mostly cardiomyocyte apoptotic bodies similar to erythrocytes.<sup>3–6</sup> Only later, when the interstitium is no longer supported by intact cardiomyocytes, may vessels become injured and give rise to genuine hemorrhage.<sup>6</sup>

If this new hypothesis is correct, where are the cardiomyocyte apoptotic bodies in Figure 2, b and d? Undoubtedly, some were already phagocytized by macrophages, many were transported away by lymphatic outflow,<sup>3</sup> and most are still in the tissue being considered to be red cells by the authors.<sup>2</sup> One may see them best in the myocardial defect in Figure 2d. There, suspended in interstitial fluid, they are constricted neither by capillaries nor by cardiomyocytes and their features may be studied without interference of these factors. Being of variable dimensions, they are practically always smaller than the

approximate diameter of cardiomyocyte nuclei in Figure 2c (4  $\mu\text{m}$ ) while red cells have the diameter of approximately 7.2  $\mu\text{m}$ . In the myocardium surrounding the defects, both individual apoptotic bodies and their conglomerates may be seen. It is difficult to reconcile large dimensions of the conglomerates with narrow interstitial spaces. In reality, the conglomerates enter into intimate contact with cardiomyocyte cytoplasm and are sometimes entirely surrounded by it. All these features indicate that the alleged red cells are cardiomyocyte apoptotic bodies. Consequently, the main mechanism of cardiomyocyte apoptotic bodies has been described in the humoral rejection of human cardiac allografts.<sup>1</sup>

Does the experimental system visualized in Figure 2, b and d, permit cardiomyocyte apoptosis to take place? The answer is yes. Cardiomyocytes possess death receptors (Fas, tumor necrosis factor receptor, etc)<sup>7</sup> and neutrophils have Fas ligand, tumor necrosis factor- $\alpha$ , etc<sup>8</sup>. In certain conditions, cardiomyocyte receptors and neutrophil ligands enter into contact resulting in cardiomyocyte apoptosis. Why hasn't this striking phenomenon been described sooner? Firstly, it may have been overlooked and secondly, it may take place only in special situations such as a deficiency of interferon- $\gamma$  in transplantation recipients.

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## Authors' Reply:

We appreciate the interest and many positive comments made by Dr. Beranek concerning our recent report investigating the rejection of MHC-mismatched cardiac allografts in the absence of IFN- $\gamma$ .<sup>1</sup> The goal of this study was to investigate mechanisms mediating the rapid rejection of organ allografts in the absence of IFN- $\gamma$ . This rapid rejection has been observed in murine models of

renal, heart, and more recently liver allografts where either the recipients are unable to produce IFN- $\gamma$  or the grafts are from IFN- $\gamma$ R1 $^{-/-}$  donors and are therefore unable to respond to IFN- $\gamma$  produced by graft-infiltrating T cells.<sup>2–6</sup> A finding that is always observed during the rapid rejection of renal, cardiac, and liver allografts in these models is severe graft tissue necrosis and hemorrhage that accompanies the rejection. Rejection of the control allografts in the presence of IFN- $\gamma$  is delayed in comparison and characterized by increasing mononuclear cell infiltration that eventually results in graft failure. Based on the necrosis observed in the absence of IFN- $\gamma$ , we hypothesized that neutrophils might play a critical role in this histopathology.

Dr. Beranek has astutely pointed out fine details of the histopathology in the allografts from IFN- $\gamma$  $^{-/-}$  recipients depicted in Figure 2 of our report. Several comments are in order regarding his evaluation of the sample sections shown. First, we were of the opinion from the first viewing that the structure shown in the lower left-hand corner of Figure 2d is not a vessel for many of the reasons stated by Dr. Beranek. Second, we did observe many small thromboses in vessels throughout the graft although these were not shown in the figure. Third, Dr. Beranek makes a good point with regard to the small bodies in the figure panel that may or may not be erythrocytes or apoptotic bodies. We have not investigated the presence or temporal aspects of myocardial apoptosis in this model. However, graft tissue hemorrhage has been observed in other solid organ allografts retrieved from IFN- $\gamma$  deficient recipients with similar histopathological features shown in Figure 2, b and d.<sup>2–6</sup> It will be of some importance to distinguish these features in the rejection of these heart allografts but as Dr. Beranek points out this may represent a very specialized case of tissue pathology. A potential solution might be the use of tissue factor staining to distinguish erythrocytes from myocardial apoptotic bodies as pointed out by Dr. Beranek in a recent letter to *The American Journal of Pathology*.<sup>7</sup> Fourth, Dr. Beranek has asked what pathological process generates the myocardial defects shown in these grafts after only 5 days. The data of the report are strongly supportive of a neutrophil mediated mechanism that occurs as rapidly as shown. In our view, this histopathology looks like neutrophil-mediated necrosis and may include neutrophil-mediated apoptosis of cardiomyocytes. It is important to state again that similar patterns of histopathology are observed in other organ allograft models in the absence of IFN- $\gamma$ .

Finally, Dr. Beranek concludes with what we feel is the most important point of this report. That is that IFN- $\gamma$  is an important regulator of early innate immune attack on the allograft. In the absence of a source of IFN- $\gamma$  the allografts are intensely infiltrated with neutrophils and quickly exhibit the tissue necrosis depicted in Figure 2 of the report. With this in mind one should be asking what regulatory aspects of IFN- $\gamma$  protect the allograft from this pathology. When we initiated these studies we had expected to observe unregulated expression of neutrophil chemoattractants (eg, KC/Groa and MIP-2) in the allografts retrieved from the IFN- $\gamma$ -deficient recipients. As



shown in Figure 5 of the report this is not the case. These results raise an important and unanswered question regarding the IFN- $\gamma$ -dependent mechanism(s) that restrict the temporal infiltration of neutrophils into the allograft to mediate this extreme histopathology. This continues to be a focus of our studies to fully understand and minimize this attack.

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## Explaining Decreased Nitric Oxide Production in Psoriatic Lesions: Arginase 1 Overexpression versus Calcitonin Gene-Related Peptide

### To the Editor-in-Chief:

I read with great interest the paper written by Bruch-Gerharz et al,<sup>1</sup> published in the January 2003 issue of *The American Journal of Pathology*. In this paper, the authors have explained the reason for the low NO concentration in the psoriatic plaques, in the face of high expression of inducible NO synthase (iNOS) mRNA and protein, by showing that arginase 1, which substantially regulates iNOS activity by competing for the common substrate L-arginine, is highly overexpressed in the psoriatic epidermis.

This is a feasible explanation, but not the only one. As a complement to the explanation for the low NO concen-

trations in psoriatic plaques, I would like to mention the effects of calcitonin gene-related peptide (CGRP) on nitric oxide generation. The pathogenesis of psoriatic plaque lesions is closely related to the overexpression of CGRP and it has been shown that CGRP-containing nerve fibers are more dense in the psoriatic epidermis.<sup>2</sup> Taylor and co-workers<sup>3</sup> have shown that CGRP suppresses the production of NO most probably through inhibition of iNOS enzymatic activity.

Therefore, it could be concluded that in addition to the overexpression of arginase 1, overexpression of CGRP in the psoriatic lesions could decrease the production of NO, thereby preventing the NO concentration to reach the keratinocytostatic levels.

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### Author's Reply:

In the letter by M. R. Namazi, the interesting idea is put forward that in addition to our demonstration of arginase 1 overexpression,<sup>1</sup> calcitonin gene-related peptide (CGRP) might contribute to depressing the iNOS activity in psoriatic plaques. His suggestion is based on two observations: a known overexpression of CGRP in psoriasis and a previous publication<sup>2</sup> on the suppressive activity of ocular aqueous humor on NO synthesis being due to the presence of CGRP.

We were well aware of these findings, however, as relates to a CGRP-mediated inhibition of iNOS activity, there are controversial data in the literature. In a series of carefully controlled experiments it has also been shown that CGRP actually enhances iNOS expression and activity<sup>3</sup> with doses of CGRP that were both lower and higher as in the first study. Moreover, in ocular aqueous humor the presence of several other factors with known and confirmed depressive action on iNOS activity had been characterized subsequently by the same group. It thus appears that depending on the presence of additional factors, CGRP may do both, either further enhance or additionally depress NO formation. And in this respect there is no way to currently estimate whether a hypothetically increased presence of this peptide in the epidermal layer might contribute to suppression of NO formation.



In contrast, there is general agreement on the influence of arginase 1 on the substrate availability for NO formation via the iNOS, and we had shown in our study that such an interaction does indeed apply for keratinocytes also. Thus, for the time being our finding serves a good and sufficient explanation for low NO formation despite iNOS expression and whether other factors hypothetically contribute (to a lesser degree) will not alter the major conclusions to be drawn, especially as concerns future therapeutic strategies.

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