Correspondence

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Cardiac Chimerism: Methods Matter

To the Editor:

Recent studies on the chimerism of the heart and other organs after sex-mismatched transplantation have provided consistent results concerning the migration of primitive cells from the host to the graft.1-5 After homing, host progenitor cells undergo replication and differentiation, generating mature parenchymal cells and vascular structures in the transplanted organ. Although there is little disagreement among authors in terms of the occurrence of this phenomenon, the magnitude of cardiac chimerism varies significantly in different reports.1,3-5 This discrepancy involves mostly ventricular myocytes and, to a lesser extent, newly formed coronary vessels. For myocytes, the published values range from as high as 18%1 to as low as 0.04%,3 or to totally absent.5

The significance of the disagreement goes beyond the physiology and pathology of the transplanted heart. The sex-mismatched transplants make it possible to document and quantitate a process that is likely to be part of normal cardiac homeostasis but it is otherwise not measurable in humans. Thus, it is not surprising that the differences in cardiac chimerism have been used to advance 2 different and competing views of heart homeostasis. We interpreted the high degree of chimerism in our specimens as further demonstration of myocyte turnover, where new myocytes replace lost ones, and as a particularly convincing proof for the formation of new myocytes in the adult human heart. Conversely, the low extent of myocyte chimerism seen by other groups has been used to question myocyte replication and to support their view that the myocardiunm is a terminally differentiated tissue incapable of undergoing meaningful regeneration.3,5 These authors have relegated the few myocytes of host origin detected in their samples to the level of curiosities without clinical or biological import. The future direction of a significant area of cardiovascular research depends on which of these 2 views of cardiac biology is correct. Therefore, we strongly believe that it is imperative to address the reasons for the apparent discrepant results published so far.

The most apparent technical difference in the 4 papers published in the last 6 months is the use of conventional light microscopy in three1-3 and confocal microscopy in one.4 Probes for the detection of the Y chromosome in female hearts transplanted into male recipients were not always the same as the procedures for the recognition of the Y chromosome. These factors introduced additional variables among these quantitative observations. In our opinion, however, the method of analysis is the major cause of the discrepancy.

An issue that raises serious technical and conceptual questions is the fact that similar high values of Y chromosome-positive myocyte nuclei were obtained by in situ hybridization of control male human hearts in 3 of the 4 studies (44%,1 67,4 and 53%).3 The most recent study did not discriminate myocytes from other cell nuclei.5 In our experience, the fraction of detectable Y chromosome in myocyte nuclei of normal male hearts by fluorescence microscopy was rather low and difficult to measure accurately. In the 6-μm tissue section illustrated here, it was 9% (Figure 1). When the same section was analyzed by confocal microscopy, the upper-optical layer of 0.6 μm showed 7% Y chromosome positive myocyte nuclei (Figure 2A). However, 50% myocyte nuclei showed the labeled Y chromosome when the full section thickness (combined projection of 10 subsequent 0.6 μm images) was examined (Figure 2B). Thus, confocal microscopy allowed us to optically section the 6-μm thick paraffin-embedded section and to count all nuclei included in the preparation and the fraction of these nuclei positive for the Y chromosome probe. Although the increase in Y chromosome-labeled cells from standard to confocal microscopy is reasonable and easy to understand, the high control values shown by conventional microscopy that were reported in 2 studies3,4 are surprising and stand in sharp contrast to the extremely low number3,4 or absence5 of Y chromosome-positive myocyte nuclei detected in the host’s heart.

The Y chromosome hybridization signal corresponds to a spheroid of 1 μm in diameter (1.02±0.31 μm; n=100) and, therefore, a

Figure 1. Light microscopic epifluorescence of a 6-μm thick section of a control male human heart (A). Y chromosome labeling of 2 myocyte nuclei (yellow rectangles a and b; total number of myocyte nuclei=22) and non-myocyte nuclei (yellow rectangles c and d; total number of non-myocyte nuclei=19) is shown by fluorescent in situ hybridization. Y chromosome positive myocyte (B and C) and non-myocyte (D and E) nuclei are also illustrated at higher magnification. Nuclei are stained by bisbenzimide (blue fluorescence), myocyte cytoplasm by α-sarcocinetic actin antibody (red fluorescence), and Y chromosome by in situ hybridization (greenish fluorescence). See text for detail. Bar=10 μm.
volume of 0.5 μm². The average volume of a myocyte nucleus is nearly 250 μm³, indicating that the labeled Y chromosome constitutes 0.2% of the entire nucleus. Under the condition in which the Y chromosome is present in a mid-section of a nucleus, the area of the Y chromosome signal is 1 μm² and the area of the nucleus profile is approximately 75 μm². The Y chromosome occupies 1.3% of the visible nucleus. It follows that the probability, P, of hitting the Y chromosome in myocyte nuclear profiles of a 6-μm thick section can be calculated to vary from a minimum of 4.4% to a maximum of 9.6%. The probability is also influenced by the orientation of myocyte nuclei in the section. These variables, including P, can be computed according to the equation: P = (d₀/2tₙ) × F, where d₀ is the diameter of the fluorescent signal (1.02±0.31 μm), tₙ is the section thickness (6 μm), and F is the average fraction of a myocyte nucleus included in the section. The value of F can be calculated from the equation: F = 1 - ([lₖ × sin(α)] + [lₙ × cos(α)])/[lₖ × sin(α)] + [lₙ × cos(α)] + tₙ), where tₙ is the nucleus transverse diameter (4.4±0.4 μm) and lₚ is the longitudinal length of the nucleus mid-section (16.8±1.7 μm). On the basis of the 2 equations, P = 4.4% for myocyte nuclei cut transversely (α=90°), and P = 9.6% for myocyte nuclei cut longitudinally (α=0°).

Because at most, 9.6% of myocyte nuclei positive for the Y chromosome can be recognized under the most favorable condition, it is surprising that in 2 studies, 53% and 67% of myocyte nuclei were found to carry the Y chromosome in light microscopic tissue sections of control male hearts. Similarly, the number of male cells identified in the female transplanted heart are not always reliable. Moreover, the capability of distinguishing adjacent structures is much greater in a 0.5-μm section than in a 6-μm thick section. This notion is confirmed by the clear recognition of the Y signal and nuclear profiles on confocal microscopy and the rather ill-defined Y label and nuclear boundary shown by light microscopy. In some cases, the Y chromosome label cannot be singled out from the nucleolus or be identified.

An important point that requires discussion relates to the emotional belief that the analysis of histochemical preparations by conventional light microscopy is superior to fluorescence labeling and confocal microscopy. As stated above, the resolution of the micrographs provided by light microscopy is markedly inferior to that obtained by confocal microscopy. Additionally, only the very superficial layer of the section can be examined by light microscopy. This inherent problem with light microscopy was recognized immediately when confocal technology became available. Clear examples were published in the Journal of Cell Biology in 1987, demonstrating the impossibility of identifying microtubules, mitotic spindle, cytoplasmic proteins, and chromosomal structures by light microscopy of cultured cells. Conversely, these morphological details were apparent when the same cells were examined by confocal microscopy. This difference between epifluorescence light microscopy and confocal microscopy is magnified in tissue sections. Today, the insistence that a conventional microscope is better than a confocal microscope is an argument that could warm the heart of the most inveterate luddite. When confocal microscopy was not available to us, the myocyte mitotic index obtained in chronic heart failure was 11 myocytes per million. When comparable hearts were evaluated by confocal microscopy, the mitotic index reached a value of 152 cells per million. Even more striking is the difference between the mitotic indices obtained by light and confocal microscopy in the border zone of acute infarcts in humans; light microscopy yielded 3.3 mitotic myocytes per million, and confocal microscopy 775 mitotic cells per million. The authors of these 3 studies were essentially the same, and the values of mitotic myocytes were collected in the same laboratory in all cases. We believe that a similar bias applies to the analysis of cardiac chimerism in the transplanted female heart.

The claim that the high percentages of Y chromosome-labeled myocytes found by us in female donor hearts was due to our inability to discriminate leukocytes or other inflammatory cells from myocytes is extravagant. In our work, specific staining for myocytes, smooth muscle cells, and endothelial cells organized in vascular structures were used to avoid misinterpretation of images. As stated, areas of rejection with inflammatory infiltrates were avoided in quantitative measurements; these sections were also negative for CD45. Moreover, distinguishing between myocytes and white blood cells is a very easy task that is made even easier by the confocal microscope. Importantly, the analysis of 0.6-μm optical sections prevented any inappropriate interpretation of myocyte nuclei, as documented in the published micrographs.

In the editorial by Taylor et al, an image obtained by light microscopy is included to claim in which manner we assigned interstitial cell nuclei to myocytes. It is extraordinary that the authors of the editorial were capable of recognizing our apparent mistakes of
interpretation of myocardial structures by using a methodology completely different from ours. It would have been more productive if they had made the effort to compare their technique with ours. We are not surprised that a procedure developed nearly 50 years ago is not as sensitive as confocal microscopy. It would have been much more appropriate for these investigators to improve their technical approach to the higher standard of the year 2002.

There is a final point that needs to be acknowledged concerning the morphometric measurement of cardiac chimerism. In view of the effects of nuclear volume and Y chromosome signal on nuclear counts, the most significant undercounting is encountered in myocytes; these cells possess large nuclei and are at times binucleated. This problem becomes less relevant for leukocytes, lymphocytes, and monocytes because of their relatively small nuclei. Similarly, these limitations are attenuated in the smaller smooth muscle cells and endothelial cells of the coronary circulation. The same principles apply to other organs, such as the liver and the intestine. For these reasons, the levels of chimerism detected in cardiac smooth muscle cells and hepatocytes and epithelial cells of the intestine were less different from the values obtained in our study.

In conclusion, confocal microscopy represents the instrumentation of choice when discrete labeling of chromosome sequences in nuclei is determined. By this technique, the portion of nuclei included in thick histological sections can be examined entirely increasing sampling and, thereby, the chances of a positive identification. This reduces sampling error; a larger sampling also improves accuracy and decreases the variability among organs in a group. These properties are particularly important in studies of chimerism in which a non-consistent approach has created significant confusion in the field, casting doubts on the magnitude and significance of progenitor cells capable of migrating from the host to the graft.

Although we are respectful of differences of opinion, believe that scientific conclusions require independent confirmation and welcome critical challenges to our results and interpretations. We also strongly feel that it is imperative that the challenger to use techniques and approaches that are at least as sensitive and discriminating as those used to obtain the observations being challenged. This is particularly important when quantitative differences are used in support or against significant biological interpretations. Disputing myocyte chimerism and its relevance undermines the notion that myocyte regeneration occurs in the adult heart and supports the dogma that the heart is a post-mitotic organ. If this effort were to be successful, in the face of increasing contrary evidence, it would seriously subvert the budding new field of cardiac stem cell biology and its exciting prospects for a new re-constitutive biological approach to post-ischemic heart disease and chronic heart failure.

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Response

As stated by Anversa and Nadal-Ginard in the accompanying letter to the editor, cardiac chimerism is an important potential paradigm shift that could impact the future direction of cardiovascular research and of treatment strategies. Because it is so important, as stated in our July 2, 2002, Circulation editorial, its proof has to be overwhelming and confirmed by multiple laboratories. The issue is not comparison and contrasting of light and confocal microscopy, which seems to be the focus of the letter by Anversa and Nadal-Ginard. Rather, as stated previously, the issue is the high frequency of artifacts due to inflammatory cells and cardiomyocyte pseudonuclei that could contribute to an incorrect interpretation of cardiomyocyte replication or chimerism. By pointing out these potential artifacts, we stressed a major issue that we feel must be directly addressed in any articles reporting cardiac chimerism, and an issue that could account, at least in part, for the discrepancy in data interpretation reported thus far. Restated, cardiomyocyte pseudonuclei, which have long been recognized both at the ultrastructural and light microscopic levels, must be directly ruled out before substantial cardiomyocyte chimerism can be accepted. To date, no data, including those in this letter to the editor, have convincingly addressed this issue. We look forward to studies that definitively establish the proportional contribution of multiple cell types (and in particular that of cardiomyocytes) to cardiac chimerism and the replication of these results by multiple groups.

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Circulation. 2002;106:e129-e131
doi: 10.1161/01.CIR.0000035926.40150.D0

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