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Quantitative Evaluation of the Rate of Myocardial Interstitial Fibrosis Using a Personal Computer

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We investigated the reliability and reproducibility of an image-analyzing system run on a personal computer for measurement of myocardial interstitial fibrosis. Measurements of myocardial interstitial fibrosis in right ventricular endomyocardial biopsies obtained from patients with hypertrophic cardiomyopathy determined by this image-analyzing system were compared with measurements determined by the point-counting method. We also investigated the correlation between measurements of interstitial fibrosis obtained by image analysis and biochemical measurements of myocardial levels of hydroxyproline in normal and cardiomyopathic hamsters. The intra- and interobserver variability were significantly lower for measurements obtained by the image-analyzing system than for measurements obtained by the point-counting system. Reproducibility was superior with the image-analyzing method. The rate of myocardial interstitial fibrosis determined by the computer image-analyzing method was positively correlated with the hydroxyproline measurement ($r=0.89$). Our results suggest that an image-analyzing system using a personal computer provides reproducible results with a high level of reliability.

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Key Words: Computer analysis; Myocardial interstitial fibrosis; Point-counting method; Hydroxyproline

The myocardium is composed of myocardial cells (parenchyma), which are contractile elements; connective tissue (interstitium), consisting mainly of collagen fibers, which are non-contractile elements; and the vasculature. Myocardial cells drive contraction and relaxation of the heart. The extracellular matrix, which is composed mainly of collagen, maintains the organization of the heart by orienting myocardial cells in the direction of long and short axes so that they contract in the same direction[1] Various stimuli can cause qualitative and quantitative changes in myocardial cells and the interstitium, resulting in a remodeling of elements of the heart[2–5] If the balance among these elements changes, cardiac function can become depressed and heart failure can occur. In studies of basic and clinical research, it is known that fibrosis of myocardial interstitium increases as a result of severe myocarditis or cardiomyopathy[6–9] Evaluation of the degree and the rate of myocardial interstitial fibrosis is important for an understanding of the etiology, effects, and prognosis of heart disease. It has been reported that in transplanted hearts the collagen content of right ventricular endomyocardial biopsies has potential value in analyzing the cause of left ventricular dysfunction[10] In the past, myocardial interstitial fibrosis has mainly been evaluated qualitatively and semiquantitatively. There are 3 quantative methods of determing the extent of myocardial interstitial fibrosis: a point-counting method[7,11,12] a method using a computer image-analyzing device[13,14] and biochemical determination of myocardial levels of hydroxyproline to estimate the amount of collagen in the myocardium[13,15] Both the point-counting method and the computer analysis method require the tissue samples. The point-counting method needs a high level of interpretative skill and is time consuming. Conventional computer analysis requires the use of expensive equipment and thus its use is limited. We investigated

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the reliability of an analysis system for measurement of the rate of myocardial interstitial fibrosis using a personal computer.

Materials and Methods

Biopsy samples were obtained from 6 patients with hypertrophic cardiomyopathy (HCM) (4 men and 2 women, aged 21−60 years, mean 45±5 years). Before admission, patients were given detailed explanations of cardiac catheter examination and right ventricular endomyocardial biopsy, and written consent was obtained. All samples were magnified 100 times under the microscope. Using the same pictures, computer analysis was compared with the point-counting method.

The other subjects were hamsters − Bio 14.6 with cardiomyopathy (n=18) and f1b with normal hamsters (n=4). All of the animals were treated according to the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. Computer analysis was compared with hydroxyproline contents as follows.

Computer Analysis of the Rate of Myocardial Interstitial Fibrosis

The computer analysis system consisted of a Macintosh LC III personal computer (Apple Computer, Cupertino, CA, USA), a Nikon COOLSCAN film scanner (Nikon, Tokyo), and Adobe Photoshop 2.01J software (Adobe Systems, Mountain View, CA, USA). Myocardial tissue samples obtained from atients with HCM were stained with Mallory azan stain and photographed with a microscope at ×100 magnification. The image was scanned into the computer using a film scanner. Myocardial cells were stained red and areas of interstitial fibrosis were stained blue (Fig 1). The number of pixels for myocardial cells and interstitial fibrosis was determined from a histogram. The endocardium itself was excluded from the analyzed field, as were blood vessels and perivascular tissue. The rate of myocardial interstitial fibrosis was determined by the following equation:

$$\text{Rate of myocardial interstitial fibrosis } (\%) = F/(M+F)\times 100$$

where M is the number of pixels indicating myocardial cells and F is the number of pixels indicating myocardial interstitial fibrosis.

The Point-Counting Method

Right ventricular endomyocardial biopsies obtained from 6 patients with HCM were stained with Mallory azan stain and magnified 100 times under a microscope. A grid constructed of equal 10-μm spaces was placed over the sample (Fig 2). More than 2,000 points of myocardial cells, which were stained red, and of interstitial fibrosis, which were stained blue, were counted. The rate of interstitial fibrosis was determined by the following equation:

$$\text{Rate of myocardial interstitial fibrosis } (\%) = F/(M+F)\times 100$$

where M is the number of points representing myocardial cells and F is the number of points indicating myocardial interstitial fibrosis.

For each method, 2 measurements were determined by each of 3 internists specializing in cardiology. We determined the variation in measurements obtained by the same observer and differences among measurements obtained by the 3 internists.

Relationship Between the Rate of Myocardial Interstitial Fibrosis Determined by the Computer Analysis and Biochemical Measurements of the Myocardial Level of Collagen

The hamsters were killed under ether anesthesia after 25 weeks. Their hearts were immediately excised and the atria and major arteries were dissected. A part of the left ventricular myocardium was obtained for histopathologic examination. The remaining myocardial tissue was used for quantitative analysis of hydroxyproline as follows. After adding to the myocardial specimens 6 mol/L hydrochloric acid in an amount equal to 100 times the wet weight, hydroxyproline was hydrolyzed by incubating the tissue for 24 h at 110 °C, and then measured by the method of Inayama et al.[16] Extracted hydroxyproline was reacted with Ehrlich's reagent and the absorbence was measured at 560 nm using a model 200-20 spectrophotometer (Hitachi, Tokyo). By using a hydroxyproline solution with known concentration, the amount of hydroxyproline contained in 1 g of myocardium was obtained from a standard curve. For histopathologic examination, the myocardial specimen obtained from the left ventricle was fixed with a 10% neutral formalin solution immediately after extraction, embedded in paraffin, and cut into 4-nm-thick slices. Paraffin-embedded slices were stained with Mallory azan stain. A total of 10−20 photographs per sample were taken with microscope at 100×magnification. Computer analysis was performed as described above. The rate of myocardial interstitial fibrosis of a sample represents the mean value obtained from 10−20

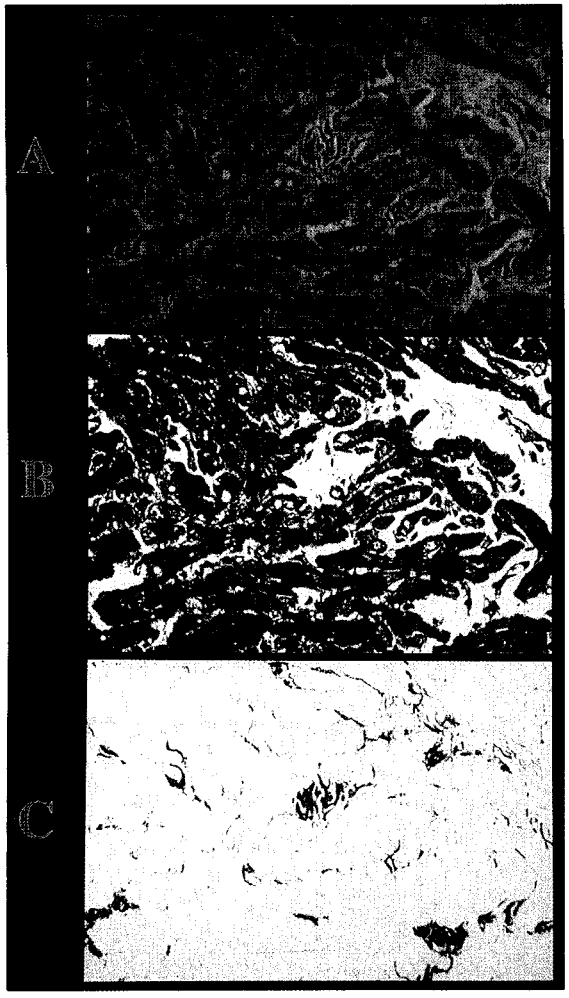

Fig 1. Computer analysis method. Light micrographs of right ventricular endomyocardial biopsy specimens (Mallory azan stain, original magnification ×100) (A). Myocytes are stained red (B) and areas of interstitial fibrosis are stained blue (C).

photographs.

Statistical Analysis

Data are shown as the means±standard deviation. Intraobserver variation was determined from the ratio of the first measurement to the second measurement. Interobserver variability was determined by comparing the ratios obtained for each internist. Data were analyzed by the F test. The correlation between the rate of myocardial interstitial fibrosis and the amount of hydroxyproline was determined using regression analysis. A p value <0.05 was accepted as statistically significant.

Results

Quantification of Myocardial Interstitial Fibrosis by the Point-Counting Method and the Computer Analysis Method

The difference in intraobserver measurements (reproducibility) obtained by the point-counting method was greater for internists A and B than for internist C, possibly reflecting differences in interpretative skill. There was no significant difference among observers in intraobserver variability of measurements obtained by the computer image-analyzing method. The difference in intraobserver measurements was significantly lower for the computer image-analyzing method than for the point-counting method (Table 1). Fig 3 gives an example of differences. Differences in measurements between observers (A/B, B/C, C/A) were significantly smaller for the computer image-analyzing method than for the point-counting method (Table 2). Fig 4 gives an example of differences.

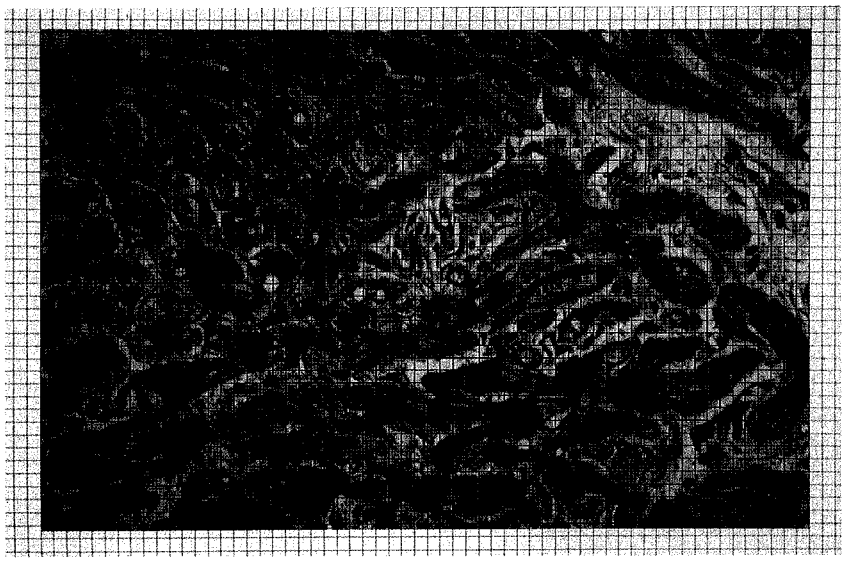

Fig 2. Point-counting method. The distance between grid points is 10 μm.

Table 1 Intraobserver Variability Ratio of Trial 1 to Trial 2

	Observer A	*Observer B*	*Observer C*
PC	*1.15±0.036*	*1.21±0.094*	*1.12±0.042*
CA	*1.02±0.015*	*1.04±0.029*	*1.02±0.033*
F test	*5.76**	*10.00**	*5.06**

PC, point-counting method; CA, computer analysis method.
*PC vs CA: *p<0.05.*

Table 2 Interobserver Ratios

	A/B	*B/C*	*C/A*
PC	*1.15±0.076*	*1.13±0.061*	*1.12±0.082*
CA	*1.03±0.016*	*1.03±0.018*	*1.03±0.027*
F test	*22.5**	*11.5**	*9.22**

A, Observer A; B, Observer B; C, Observer C; PC, point-counting method; CA, computer analysis method.
*PC vs CA: *p<0.05.*

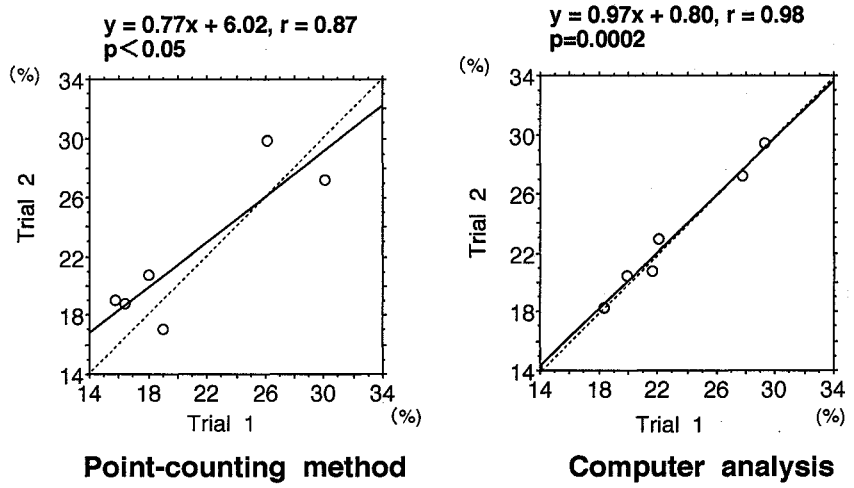

Fig 3. Intraobserver variability.

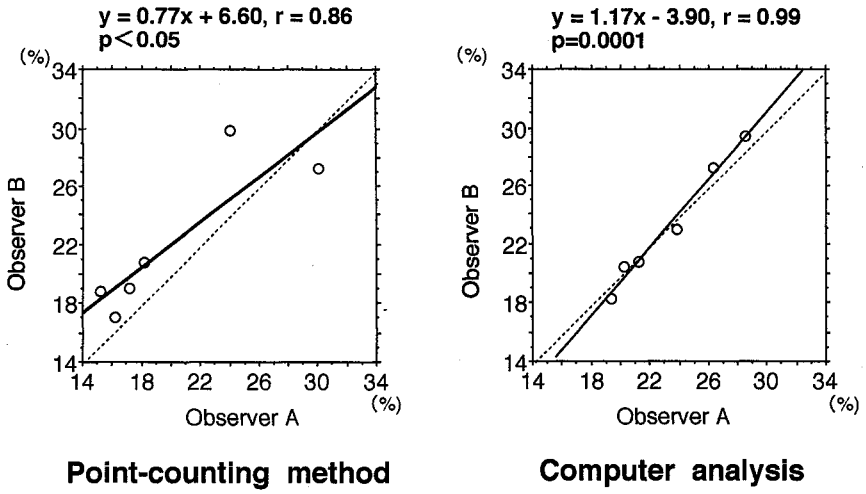

Fig 4. Interobserver variability.

Relationship Between Measurements of Myocardial Interstitial Fibrosis Obtained by the Computer Analysis and the Amount of Hydroxyproline

The rate of myocardial interstitial fibrosis determined by the computer image-analyzing method was significantly correlated with hydroxyproline measurements (Fig 5, r=0.89).

Discussion

Evaluation of the rate of myocardial interstitial fibrosis falls into 2 broad categories: qualitative evaluation and quantitative evaluation. In the qualitative evaluation method, tissue images are graded according to the patterns observed when visualized under a

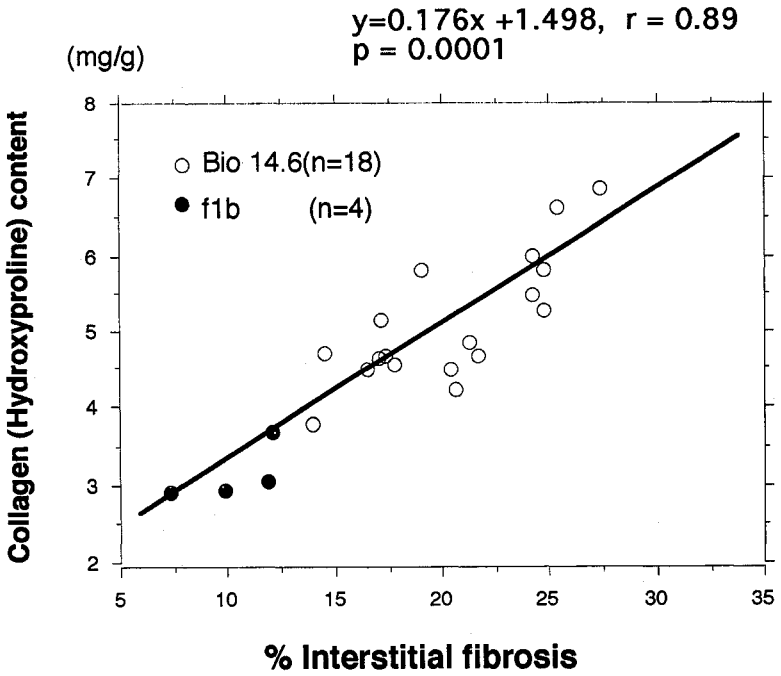

Fig 5. Relationship between the percentage of interstitial fibrosis determined by the computer analysis method and collagen (hydroxyproline) content.

microscope[17,18] However, these qualitative methods do not accurately estimate the severity of myocardial lesions. Quantitative evaluation methods include the point-counting method[7,11,12] a computer-analysis method[13,14] and measurement of the myocardial level of hydroxyproline[13,15] The point-counting method, which can be applied to both biopsy and autopsy specimens, requires measurement of a large number of points. Sugihara et al[7] reported that, for accuracy, it was necessary to measure at least 2,000 points. The point-counting method is also time and labor consuming, and is influenced by subjective variations in measurements. In the present study, intraobserver variability in measurements obtained by the point-counting method was greater for the inexperienced than for the skilled internist. Measurements obtained by the image-analyzing system were not affected by the internist's skill. The difference in measurements obtained by the same internist was significantly smaller for the image-analyzing system than for the point-counting method, and the image-analyzing system demonstrated good reproducibility. Measurement of 1 sample took 10 min with the image-analyzing system compared with 60 min for the point-counting method. Thus, it was considered extremely effective from the point of time efficiency.

Digitizing images using a computer is the equivalent of placing a grid over the specimens in the point-counting method. With a film scanner, the number of pixels that make up the image can be controlled by varying the pitch of the scanner. The sensor of the scanner used in the present study has a capacity of 2,592×3,888 pixels. We scanned the film at the rate of about 450 dots per inch. Thus, a 24.3×36.5 mm film was converted into 432×648 pixels. However, strips are continuous and further investigation of accuracy of resolution in terms of the number of pixels is needed. Computer images can be processed to appear as red, green, and blue (RGB method). The personal computer used in the present study can express the gradation of each of 3 primary colors from 0 to 255 (8 bits). Thus, each color has 256 gradations giving a total of 16.7 million different expression types. We used a software application for image editing that is capable of processing and outputting digitized images in various ways. We were able to select portions of the same color if we wanted to digitize an arbitrary portion of the image. In addition, the total number of pixels in the selected area could be displayed. Although previous imaging systems have used expensive image-processing devices designed exclusively for this purpose, the software we used appeared to display color gradations adequately.

Biochemical measurements of hydroxyproline used to estimate the myocardial level of collagen[13,15] provide accurate determinations, but are not usually used because of the small size myocardial biopsies. In the present study, measurements of myocardial interstitial fibrosis obtained by the computer image-analyzing system were significantly correlated with biochemical

determinations of the myocardial level of hydroxyproline.

In conclusion, our results showed that the image-analyzing system using a personal computer demonstrated good reproducibility and reliability for measurement of myocardial interstitial fibrosis.

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