Functional Abnormalities of Circulating Natural Killer Cell Subpopulations in Patients with Dilated Cardiomyopathy

TSUGIYASU KANDA, TOMOYUKI YOKOYAMA, TADASHI SUZUKI and KAZUHIKO MURATA

The Second Department of Internal Medicine, Gunma University School of Medicine, Maebashi, Gunma 371

KANDA, T., YOKOYAMA, T., SUZUKI, T. and MURATA, K. *Functional Abnormalities of Circulating Natural Killer Cell Subpopulations in Patients with Dilated Cardiomyopathy.* Tohoku J. Exp. Med., 1992, 168 (3), 529-537 — We investigated abnormalities in natural killer (NK) cells in the myocardium and circulating blood of 38 patients with idiopathic dilated cardiomyopathy (DCM), 18 patients with hypertrophic cardiomyopathy, 8 patients with primary amyloidosis, and 12 age-matched normal control subjects. Immunohistochemical staining of myocardial biopsies revealed a significantly greater number of CD57-positive NK cells in patients with DCM than that in controls (3.7 ± 2.7 v.s. 1.9 ± 1.6 , $p < 0.05$). The New York Heart Association functional class, left ventricular ejection fraction, myocardial fiber diameter, and interstitial fibrosis volume fraction did not differ significantly between the DCM patients who died within five years of diagnosis and the 31 surviving DCM patients. However, there were significantly fewer CD57-positive NK cells in patients who died than in surviving patients ($p < 0.05$). There were no significant differences in the peripheral NK cell activity or the number of NK subset cells between the 16 patients with DCM ($n = 16$) and the 12 age-matched normal controls. In normal controls, the number of some NK cell subpopulations ($CD16^+$, $CD57^+$, $CD16^+ CD57^+$, and $CD8^+ CD57^+$ cells) were positively correlated with NK cell activity. In patients with DCM, there was no correlation between the number of NK cell subpopulations and NK cell activity. Our findings indicate that functional abnormalities exist in NK cell subpopulations in patients with DCM, and that these abnormalities may be related to the pathogenesis of DCM. ——— natural killer cell; immunohistochemistry; NK cell activity; dilated cardiomyopathy

Viral infections and immune responses are believed to contribute to the myocardial injury associated with idiopathic dilated cardiomyopathy (DCM). Investigators have focused on the role of natural killer (NK) cell activity in the host defenses against viral infection. NK cells have been found to synthesize cytotoxic mediators, which may lead to myocardial injury associated with myocarditis and DCM. NK cells have been found to infiltrate the myocardium, suggesting that they may produce cell damage in patients with myocarditis (Young et al. 1990). However, the role of the NK cells in damaged myocardium

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and NK cell subpopulations in peripheral blood has not been investigated in DCM.

In this study, we immunohistochemically examined NK cells in formalin-fixed, paraffin-embedded myocardial biopsy specimens and compared the NK cell activity and NK cell subpopulations in the peripheral blood between patients with DCM, patients with coronary heart disease, and age-matched controls to identify quantitative and qualitative abnormalities associated with DCM.

MATERIALS AND METHODS

Histological study: We studied 38 patients with DCM (mean age: 46 ± 9.6 years; range: 21–68; 31 men, 7 women), all of whom underwent cardiac catheterization to exclude coronary artery and valvular heart disease; 18 patients with hypertrophic cardiomyopathy (HCM) (mean age: 45.9 ± 6.3 years; range: 38–66; 13 men, 5 women); 8 patients with primary amyloidosis (mean age: 58.0 ± 6.8 years; range: 38–66; 5 men, 3 women) as one of secondary cardiomyopathy; and 12 normal control subjects (mean age: 48.9 ± 11.0 years; range: 33–58; 8 men, 4 women) with a diagnosis of arrhythmia.

Myocardial biopsy specimens were obtained from the interventricular septum of the right ventricle using a Konno or Cordis endomyocardial biptome, which was introduced via the saphenous vein in the inguinal region and guided into the right ventricle under fluoroscopic control. Samples were immediately placed in a 3% buffer solution and embedded in paraffin wax for histological examination. Myocardial sections were stained routinely with hematoxylin and eosin, periodic acid-Schiff, and Mallory's stain for collagen. Myocardial fiber diameter was determined at a magnification of $\times 160$; interstitial fibrosis volume fraction was determined at a magnification of $\times 400$. The fiber diameters were measured using an eyepiece micrometer. The average minimum diameter of 20 cross-sectioned fibers in each biopsy sample and the volume of interstitial fibrosis were measured

Fig. 1. CD57-positive natural killer cells (arrows) ($\times 240$).

by light-microscopic morphometry.

For immunohistochemical study, 4- μ m thick serial sections were mounted on albumin-coated glass slides and deparaffinized. Intrinsic peroxidase was inactivated by using 3% H_2O_2 in methanol. Sections were then incubated with anti-CD57 (formerly Leu 7) monoclonal antibodies (1:100 dilution; Becton Dickinson, Inc., Mountain View, CA, USA) (Lanier et al. 1983), washed with phosphate-buffered saline, and incubated with avidin-biotin peroxidase complex (BioGen Laboratories, Dublin, CA, USA). For light microscopic examination, the sections were treated with diaminobenzidine-peroxidase solution for 7 min, dehydrated, and counterstained with Mayer's hematoxylin. We counted the number of CD57-positive cells per high-powered ($200\times$) microscopic field (Fig. 1). Three sections in each specimen and at least five fields per section were examined, and the mean number of CD57 cells was determined.

NK cell subsets and NK cell activity in peripheral blood. To investigate the relation between circulating NK cell subpopulations and DCM, we studied 16 patients with DCM (mean age: 48.0 ± 8.3 years), 16 patients with coronary heart disease (CHD), and 12 age-matched healthy subjects. NK cell subsets and NK cell activity were determined using a previously described method (Yu et al. 1984). Briefly, blood was anticoagulated with sodium citrate, and lymphocytes were separated by Ficoll-gradient centrifugation. NK subpopulations were identified by sequential positive selection using anti-CD57 and anti-CD16 (formerly Leu 11a) monoclonal antibodies (Becton Dickinson Inc.) with an FCM-1D dual-color analyzer. NK cell activity was determined using standard procedures (Kanda et al. 1990). Briefly, lymphocytes were incubated with ^{51}Cr -labeled K562 targets at various ratios (1:1 to 25:1) for 16 hr. Specific release of ^{51}Cr into the supernatant was assayed with a radioimmunoassay kit (Eiken, Tokyo).

Statistical analysis. Data are reported as mean $\pm$ standard deviation (s.d.). Statistical comparisons between groups were made using the Student's *t*-test. A *p* value < 0.05 was considered to be statistically significant.

RESULTS

Immunohistological analysis

Analysis of myocardial biopsy specimens revealed a significantly greater number of CD57⁺NK cells in DCM patients than in controls (mean: 3.7 ± 2.7 vs. 1.9 ± 1.6 , $p < 0.05$). The number of CD57⁺NK cells was slightly, but not significantly, elevated in HCM and amyloidosis patients compared with control subjects (Fig. 2).

New York Heart Association (NYHA) functional class, left ventricular ejection fraction, or morphometric findings, myocardial fiber diameter and interstitial fibrosis volume, did not differ between DCM patients who died within 5 years of biopsy ($n=7$) and those who survived ($n=31$). Immunohistochemical analysis showed that the number of CD57⁺NK cells was significantly lower in the patients who died than in those who survived ($p < 0.05$) (Fig. 3).

Relation between circulating NK cell subsets and NK cell activity.

There were no significant differences in the number of NK subpopulations (CD16⁺, CD57⁺, CD16⁺CD57⁻, CD16⁺CD57⁺, CD16⁻CD57⁺, and CD8⁺CD57⁺ cells) or NK cell activity among the patients with DCM ($n=16$), the patients with CHD ($n=16$), and the controls ($n=12$) (Table 1). In the control group, the

Fig. 2. The number of CD57⁺ natural killer (NK) cells per high-power field ($\times 200$, HPF) in myocardial biopsy specimens from patients with dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), and cardiac amyloidosis (Amyl) and controls (cont). * $p < 0.05$.

number of CD16⁺, CD57⁺, CD16⁺CD57⁺ and CD8⁺CD57⁺ cells was significantly correlated with NK cell activity. In CHD patients, the number of CD16⁺, CD16⁺CD57⁺, CD16⁺CD57⁺ and CD8⁺CD57⁺ cells was correlated with NK cell activity (Fig. 4). In patients with DCM, only the CD16⁺CD57⁺ NK cell subpopulation was correlated with NK cell activity (Table 2). The number of CD16⁺CD57⁺ cells was not correlated with NK cell activity in any of the groups. The number of CD57⁺ NK cells in myocardial biopsy specimens from patients with DCM ($n=5$) was not proportional to either the circulating NK cell subsets or the NK cell activity.

DISCUSSION

Our results showed that NK cells were increased in endomyocardial biopsy specimens from DCM patients compared with normal controls. A decreased number of NK cells were observed in patients with DCM who died within 5 years compared with surviving patients. No significant differences were observed in functional class, left ventricular ejection fraction, or histological findings between these two DCM patient groups. The activity of NK subpopulations in peripheral blood appeared to be impaired in patients with DCM compared with CHD patients or normal subjects.

Linder and McManus (1985) identified a higher number of CD57⁺ cells ($2.7 \pm 1.0/\text{mm}^2$) in right ventricular endomyocardial biopsy specimens from DCM patients than in controls ($0.4 \pm 0.2/\text{mm}^2$). In our study, DCM patients exhibited a higher number of CD57⁺ NK cells than did HCM patients, primary amyloidosis patients, and controls. To date, no functional studies of myocardial NK cells

Fig. 3. Clinical and histological characteristics of patients with dilated cardiomyopathy. 1) New York Heart Association (NYHA) functional class; 2) left ventricular ejection fraction; 3) number of natural killer (NK) cells/high power field (HPF) ($\times 200$); 4) myocardial fiber diameter; 5) interstitial fibrosis volume. Alive, surviving patients; Dead, patients who died within 5 years of diagnosis.

* $p < 0.05$; NS, not significant.

TABLE 1. NK cell subpopulations and NK cell activity in patients with dilated cardiomyopathy (DCM, $n = 16$), coronary heart disease (CHD, $n = 16$) and normal controls (NC, $n = 12$)

	NK cell subpopulation (%)						NK cell activity (%)
	CD16	CD57	CD16 ⁺ CD57 ⁻	CD16 ⁺ CD57 ⁺	CD16 ⁻ CD57 ⁺	CD8 ⁺ CD57 ⁺	
DCM	14.8 ± 6.0	23.0 ± 13.3	3.2 ± 1.2	11.6 ± 4.8	8.2 ± 7.3	11.4 ± 7.2	22.3 ± 10.6
CHD	15.0 ± 12.3	24.4 ± 12.9	4.9 ± 5.2	10.1 ± 7.1	6.9 ± 7.7	11.8 ± 10.9	21.8 ± 12.2
NC	15.1 ± 9.7	25.2 ± 11.2	5.2 ± 2.8	9.9 ± 6.9	9.0 ± 4.9	14.2 ± 6.5	29.0 ± 11.2

Fig. 4. Correlations between the number of CD16⁺ NK cells and NK cell activity in dilated cardiomyopathy (DCM), coronary heart disease (CHD), and normal controls (NC).

TABLE 2. Correlations between NK cell subpopulations and NK cell activity in circulating blood of patients with dilated cardiomyopathy (DCM), coronary heart disease (CHD), and normal controls (NC). Underlines indicate a significant correlation ($p < 0.05$)

	NK cell subpopulation					
	CD16	CD57	CD16 ⁺ CD57 ⁻	CD16 ⁺ CD57 ⁺	CD16 ⁻ CD57 ⁺	CD8 ⁺ CD57 ⁺
DCM	0.55	0.43	-0.02	<u>0.63</u>	0.32	-0.11
CHD	<u>0.68</u>	-0.03	<u>0.57</u>	<u>0.68</u>	0.47	<u>0.91</u>
NC	<u>0.79</u>	<u>0.57</u>	<u>0.67</u>	<u>0.81</u>	0.50	<u>0.69</u>

have been published. NK cells contain several cytotoxic mediators, such as perforin (Young et al. 1990), that may accelerate the DCM-associated myocardial injury. In humans, CD57⁺ lymphocytes are believed to function as active cytotoxic cells (Abo and Balch. 1982). There are several possible reasons why CD57⁺ cells were decreased in the myocardium of DCM patients with a poor prognosis. The patients with a poor prognosis may be immunosuppressed. NK cells *in situ* may confer protection against the destruction by virus or external agent. Treatment with an immunomodulator OK432 was found to increase the numbers of NK cells in the myocardium of mice and to protect the animals against viral myocarditis (Yokoyama et al. 1991). It is possible that there are at least two etiologic types of DCM; one is a sequela to an earlier viral myocarditis and the other is a cardiomyopathy of unknown origin with a poor prognosis. A significantly increased number of large mononuclear cells (one of the NK cell subpopulations) has been observed in the convalescent stages of myocarditis (Kanda et al. 1990). Levi et al. (1977) reported that patients with coxsackievirus myocarditis have a good prognosis. Another possibility is that damaged myocardium with intercellular fibrosis may have a reduced interstitial space and consequently a reduced capacity to manufacture lymphocytes.

NK cell function appears to be an important first line defense against viral infection and to provide immunosurveillance against malignancy. The NK cell activity in the peripheral blood of patients with DCM has been reported to be low (Linder et al. 1985). However, our results showed that NK cell activity and the number of NK cells did not differ between patients with DCM and controls. Correlations between NK cell activity and NK cell subsets (CD16⁺, CD57⁺, CD16⁺CD57⁻, CD8⁺CD57⁺ cells) differed among the patients with DCM, patients with CHD, and normal controls. NK cell subsets except for CD57⁺ cells, had a significant correlation with NK cell activity in controls and in patients with CHD. In DCM patients, the percentages of NK cell subpopulations were correlated with NK cell activity. The CD16⁺CD57⁻ NK cells have been found to have the greatest degree of cytotoxicity, followed by CD16⁺CD57⁺ and CD16⁺CD57⁻ (Lanier et al. 1983). The CD8⁺CD57⁺ NK subpopulation is a natural suppressor, which produces immunosuppressive lymphokines (Hertel-Wulff and Strober. 1988). Itagaki et al. (1988) described a case of DCM with depressed NK cell activity and no correlation between NK cell activity and the number of CD57⁺ NK cells. Differences in the NK cell subpopulations between the DCM and CHD patients do not appear to be related to an increased activity of the sympathetic nervous system because their NYHA classification is similar (Kanda et al. 1990). A poor correlation between NK activity and NK cell subsets has been reported in patients with advanced multiple myeloma (Osterbarg et al. 1990) and those with malignant neoplasms (Sorskaar et al. 1986). These findings point to an immune related etiology for DCM and suggest that there is an association between disease with abnormal immune regulation, perhaps in conjunction with an infectious

process.

We did not observe a significant correlation between the number of CD57⁺ NK cells in myocardial biopsy specimens and the NK subpopulation in the peripheral blood of DCM patients. We did not detect the CD16 antigen in formaldehyde fixed and paraffin-embedded specimens either. The CD16 antigen appeared to be modified during the routine fixation and embedding. An accurate measurement of NK cells can only be obtained in autopsied myocardium because a biopsy sample provides limited data. However, quantitative analysis of NK cell infiltration provides important diagnostic and therapeutic information and the transverse endomyocardial biopsy is a safe procedure associated with a low morbidity. Further functional studies of NK cells *in situ* will help to clarify the relation between the number of NK cells and myocardial injury.

Our findings suggest that quantitative increase in NK cells in the myocardium and qualitative abnormalities of NK cell subpopulations in the peripheral blood may be related to the pathogenesis of DCM. Whether NK cell dysfunction is a cause or an effect of DCM remains to be determined.

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