GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Hydroxyproline Content and Fibrogenesis in Mouse Liver and Lungs during the Early Stages of BCG Granulomatosis

V. A. Shkurupiy*,, L. B. Kim*, I. K. Nikonova*, O. V. Potapova*, L. A. Cherdantseva*, and T. V. Sharkova***

Translated from *Byulleten' Eksperimental' noi Biologii i Meditsiny*, Vol. 154, No. 9, pp. 273-277, September, 2012
Original article submitted June 1, 2011

In generalized BCG granulomatosis, fibrosis starts early (on day 3) and not only around the granulomas, but also in the organs. The severity of organ fibrosis is apparently determined by the concentration of granulomas, in particular their macrophages inducing proliferation of fibroblasts in organs and granulomas as well as activation of fibrogenesis. On day 30 after infection, the degree of fibrosis in the lungs was by 6 times higher than in the liver. The increase in hydroxyproline concentration in organs in early period of infection was determined by acute stress, while on day 30 it resulted from its enhanced synthesis by granuloma fibroblasts and resident fibroblasts in organs.

Key Words: *BCG granulomatosis; hydroxyproline; liver, lungs, morphometry*

Connective tissue homeostasis in different organs is maintained by fibroblasts. The number, concentration, and synthetic and catabolic activity of fibroblasts are apparently determined by structural features of the organs. For example, the amount of the connective tissue differs significantly in the liver and lungs. We can assume that in case of damage, growth factors synthesized by macrophages in the regenerative phase of inflammation [3,8,12] in the focus of inflammation will trigger collagen synthesis by fibroblasts outside the damaged area. If so, fibrosis in generalized inflammation would be more pronounced in the organ with greater concentration of fibroblasts. It was shown that in generalized, *e.g.* BCG-induced granulomatosis, the so-called granulomas with high concentration of macrophages and collagen-synthesizing fibroblasts appear in all organs one month after infection. However, reparation processes had not yet begun in organ parenchyma in areas damaged by mycobacterial toxins [5]. Granuloma macrophages triggering collagen synthesis by fibroblasts in granulomas [5] would presumably induce collagen synthesis by organ fibroblasts. In this case, fibroblast concentration in the organ will affect the degree of general fibrosis regardless of the processes of defective repair (*substitucio*) at damaged parenchymal sites or, in case of BCG granulomatosis, in granulomas.

Here we studied liver and lung fibrosis during the early stages of BCG granulomatosis before the development of replacement fibrosis at the sites of parenchymal damage.

MATERIALS AND METHODS

The study was performed on 2-month-old male BALB/c mice weighing 20-22 g obtained from the nursery of Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences. The

*Research Center of Clinical and Experimental Medicine, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk; **Novosibirsk State Medical University, Russia. ***Address for correspondence:*** lenkim@soramn.ru. L. B. Kim

0007-4888/13/1543-0299

mice were maintained on a standard laboratory diet with free access to food and water. BCG granulomatosis was simulated in mice (n=20) by intravenous (retroorbital) injection of BCG in a dose of 0.5 mg/kg body weight in 0.85% NaCl. Control mice (n=10) received 0.85% NaCl. The animals were sacrificed by cervical dislocation under ether anesthesia (days 3 and 30 postinfection) and liver and the lungs were sampled. For histological study, organ samples were fixed in 10% neutral formalin and embedded in paraffin. Sections (4-5 μ) were sliced on a HM355S microtome (Microm), stained with hematoxylin and eosin and by van Gieson's method, and examined under an AxioImager A1 light microscope equipped with an AxioCam MRc5 digital camera using AxioVision software (rel. 4.8; Carl Zeiss). For morphometric studies, a closed test system was used consisting of 100 evenly spaced sample points in the area of 3.64×10^5 μ^2.

For biochemical studies, the liver and the lungs were removed, weighed, and then deep frozen. Hydroxyproline was assayed [9] in the liver and lungs of the above mouse groups and in intact strain, gender, and age-matched mice (n=10). Optical density of the samples was measured at 550 nm on a PD-303S Apel spectrophotometer.

The data were statistically processed using Statistica 10.0 software (Statsoft). Depending on the nature of the distribution, nonparametric Mann–Whitney test and Student t test were used to determine significant differences between the compared means with an accepted significance level of $p<0.05$.

RESULTS

On day 3 after infection, perivascular infiltrates were found, which consisted mainly of mononuclear cells. Similar infiltrates were located perivascularly and peribronchially in the lungs. Numerical density (concentration) infiltrates in the lungs was about 2 times higher than in the liver (Table 1). At that time, more than 20% hepatocytes underwent vacuolar degeneration, whereas no such alterations were observed in the lungs. On day 30, typical BCG granulomas were formed in the liver and lungs. Their numerical density in the lungs was 2.4 times higher than in the liver, but the size (diameter) was less (Table 1). During this period, over 50% hepatocytes were in the state of vacuolar degeneration while in the lungs only some cells of the alveolar epithelium underwent degenerative changes (Table 1). Necrotic cells constituted only ~4% of the liver parenchyma and less than 1% of the lung parenchyma. In none of the organs, signs of repair at the site of damage such as incomplete regeneration (presence of collagen fibers) were observed. At the same time,

TABLE 1. Structural Changes in the Liver and Lungs of BALB/c Mice after Intravenous Infection with BCG ($M\pm m$)

Parameter	Day after BCG infection	Liver	Lungs
Numerical density of BCG granulomas	3	0.78±0.08	1.48±0.18*
	30	3.08±0.16	7.40±0.57*
Diameter of BCG granulomas, μ	3	4.27±0.42	6.21±0.42*
	30	47.25±1.26	39.69±1.24*
Volume density of degeneration, %	3	24.72±1.18	-
	30	50.18±1.22	2.64±0.24*
Volume density of necrosis, %	3	-	-
	30	3.75±0.24	0.75±0.12*
Volume density of tissue collagen fibers	3	0.15±0.06	0.36±0.18
	30	2.86±0.30	5.22±1.08*
Volume density of granuloma collagen fibers	3	-	0.18±0.08
	30	4.27±0.60	4.08±0.24
Numerical density of granuloma fibroblasts	3	1.26±0.53	1.45±0.53
	30	2.34±0.20	7.57±0.56*
Index of fibroplastic activity in granuloma fibroblasts	3	-	0.40±0.07
	30	1.82±0.18	0.53±0.48*

Note. *$p<0.05$ in comparison with the corresponding parameter in the liver; "–": absence of the object of research or phenomenon.

the formation of collagen fibers unrelated to granulomas in the liver (periportally and perivascularly) and in the lungs (perivascularly and peribronchially), but not in areas of parenchymal destruction was observed. Volume density of collagen in the lungs was about two-fold greater than in the liver on experimental day 3 (Table 1). The data obtained on day 3 of the experiment can be neglected due to low content of collagen in both organs. The data also indicate high sensitivity of hepatocytes to *M. tuberculosis* metabolic products compared with pulmonary epithelial cells. They also point to the fact that the role of activated oxygen species generated by activated granuloma macrophages in the organ damage [2] is probably overestimated, particularly in tuberculosis, because in the lungs with granuloma concentration 2.4 times higher than in the liver, the degree of destructive processes was substantially lower (Table 1). Nevertheless, the volume density of granuloma collagen (percentage of the granuloma volume) was similar in both organs on day 30. However, if the concentration of fibroblasts in granulomas was the same in both organs on day 3, it was more about 3 times in the lung granulomas on day 30 (Table 1). At the same time, index of fibroplastic activity of fibroblasts (granuloma collagen per 1 fibroblast) was 3 times lower in lung granulomas than in the liver (Table 1). If the amount of collagen per 1 granuloma was the same in both organs, while the size of granulomas in lungs was less and their numerical density was 2.4 times higher than in the liver, the collagen concentration in lung granulomas was also 2.4 times higher. In the lung, the concentration of collagen not associated with granulomas per organ volume unit was also 2 times higher than in the liver. These data suggest that the same factors determining granuloma

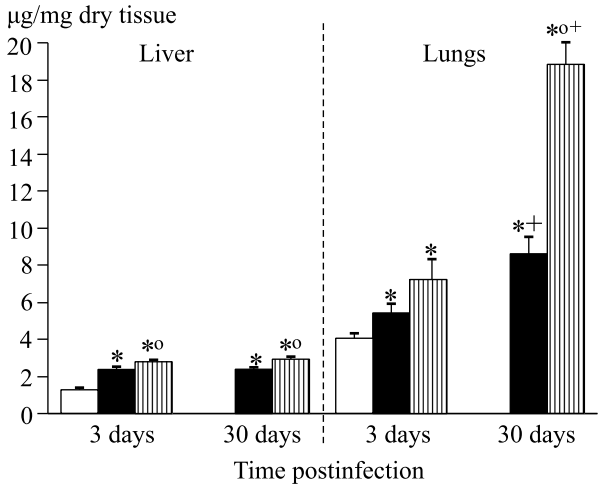

Fig. 1. Hydroxyproline content in the liver and lungs of BALB/c mice after intravenous infection with BCG. Light bars: intact mice; dark bars: control mice; hatched bars: infected mice. $p<0.05$ in comparison with *intact mice, °controls, +day 3.

fibrosis induce collagen synthesis by external fibroblasts. They are obviously related to increased amount of macrophages in the organs and secretion of growth factors stimulating fibroblast proliferation and collagen synthesis [6,15]. Fibroblast proliferation was more active in lung granulomas, but the fibroblasts showed substantially lower mean fibroplastic activity there. It was possibly linked with the fact that proliferating fibroblasts contain the smaller fraction of differentiated cells capable to synthesize collagen. The wide variation in the mean index of fibroplastic activity of lung granuloma fibroblasts on day 30 after infection supports this assumption (Table 1). Biochemical study on hydroxyproline determination characterizes the dynamics of metabolism (synthesis and degradation of collagen) in the organs. Some authors consider hydroxyproline as a marker of collagen content [4]. It is believed that increased hydroxyproline content in the organs (liver) reflects the severity of fibrosis [14] and collagen amount. However, increased hydroxyproline may be due both to collagen synthesis and degradation. Structural collagen is abundant in the lungs, but not in the liver. It is quite difficult to determine the source of hydroxyproline, especially in this situation, since we know that *M. tuberculosis* and their lipoarabinomannan stimulate activation and secretion of zinc-containing matrix metalloproteinases in macrophages [7,13]. This can be associated with accumulation of hydroxyproline. In addition, there is another pathway coupled with the induction of IL-1β and TNF-α by macrophages inducing increased synthesis and secretion of matrix metalloproteinases by fibroblasts. On the other hand, activation of macrophages, particularly in granulomas, can be associated with proliferation and activation of fibroblast collagen synthesis. Thus, the presence of tenascin-C, protein precursors of type I and type III collagen, and myofibroblasts was shown around the granulomas [11]. According to these data, type I collagen precursor was expressed both within the granulomas. According to our data, hydroxyproline content in the lungs of intact animals was 3 times higher than in the liver (Fig. 1). In the experiment after infection of animals, lung hydroxyproline levels did not differ on days 3 and 30, but exceeded these by 15 and 20% in time-matched controls (Fig. 1). Morphological study indicates the balance between hydroxyproline synthesis and utilization during the formation of collagen. Its production was slightly ahead of "consumption", because it was "consumed" for the formation of the fibrous skeleton of granulomas and portal collagen. In general, hydroxyproline content was significantly higher both in control animals treated with 0.85% NaCl and in animals infected with *M. tuberculosis* compared with intact animals. This may be associated with acute stress [1]. The destruction of

organ collagen in control animals is possible due to activation of resident macrophages by stressful procedure (NaCl infusion) [10] and secretion of matrix proteases by these cells.

In the lungs, the content of organ collagen is much higher than in the liver. In control animals receiving isotonic NaCl, the absolute "post-stress" hydroxyproline content in the lungs was significantly higher than in the liver, especially on day 30. Hydroxyproline concentration in the lungs of infected mice increased by more than 25% compared to controls on day 3, and 116% on day 30 (Fig. 1). It may be assumed that the significantly increased lung hydroxyproline compared with that of the liver is associated with the fact that the content of fibroblasts is significantly higher in the lungs than in the liver, and pulmonary granuloma fibroblasts showed fibroplastic activity 3 times lower than these in the liver. Pulmonary fibroblasts activated by granuloma macrophages provided the high level of collagen synthesis. Their concentration in these organs was 2 times higher than in the liver (Table 1). In this case, the volume density of collagen outside the granulomas was almost 2 times higher than in the liver (Table 1).

Thus, according to our data, hydroxyproline content increased in both organs in the early stages of BCG granulomatosis, when granulomas and destructive processes were formed in the liver and lungs, but reparative processes implying replacement of lost parenchyma by the fibrous connective tissue were still absent in the areas of parenchymal destruction. Hydroxyproline can originate from either post-stress disintegration of organ collagen, or be synthesized by granuloma fibroblasts and by organ fibroblasts, which were apparently activated by granuloma macrophages. Therefore, we can hypothesize that generalized tuberculosis granulomatosis promotes early organ fibrosis not only around the granulomas. In this experimental situation, the degree of organ fibrosis is probably determined by concentration of granulomas (their fibroblasts and macrophages) as well as by activation of proper organ fibroblasts and their fibroplastic activity.

REFERENCES

1. N. N. Vasilieva, E. V. Yeliseyeva, L. S. Isakova, *et al*., *Vestn. Novykh Med. Tekhnol*., No. 1, 27-29 (2002).
2. V. A. Krasnov, N. K. Zenkov, A. R. Kolpakov, *et al*., *Probl. Tuberkul. i Bol. Legkikh*, No. 9, 9-17 (2005).
3. D. N. Mayansky and I. G. Ursov, *Lectures on Clinical Pathology: A Guide for Physicians* [in Russian], Novosibirsk (1997).
4. N. P. Omelyanenko and L. I. Slutskiy, *Connective Tissue (Histophysiology and Biochemistry)* Vol. 1, [in Russian], Ed. S. P. Mironov, Moscow (2009).
5. V. A. Shkurupiy, *Tuberculous Granulomatosis. Cytophysiology and Targeted Therapy* [in Russian], Moscow (2007).
6. R. Bataller and D. A. Brenner, *J. Clin. Invest*., **115**, No. 2, 209-218 (2005).
7. J. C. Chang, A. Wysocki, K. M. Tchou-Wong, *et al*., *Thorax*, **51**, No. 3, 306-311 (1996).
8. S. Chujo, F. Shirasaki, S. Kawara, *et al*., *J. Cell. Physiol*., **203**, No. 2, 447-456 (2005).
9. C. A. Edwards and W. D. O'Brien, *Clin. Chim. Acta*., **104**, No. 2, 161-167 (1980).
10. R. Erdosova and M. Kraus, *Physiol. Bohemoslov*., **25**, No. 1, 1-6 (1976).
11. R. Kaarteenaho-Wiik, O. Sademies, P. Paakko, *et al*., *Hum. Pathol*., **38**, No. 1, 147-153 (2007).
12. A. Leask and D. J. Abraham, *Biochem. Cell. Biol*., **81**, No. 6, 355-363 (2003).
13. J. L. Taylor, J. M. Hattle, S. A. Dreitz, *et al*., *Infect. Immun*., **74**, No. 11, 6135-6144 (2006).
14. A. Wangoo, I. N. Brown, B. G. Marshall, *et al*., *Clin. Exp. Immun*., **119**, No. 1, 92-98 (2000).
15. B. C. Willis, R. M. du Bois, and Z. Borok, *Proc. Am. Thorac*., **3**, No. 4, 377-382 (2006).