DOI 10.1007/s10517-015-2752-9

Serum, Liver, and Lung Levels of the Major Extracellular Matrix Components at the Early Stage of BCG-Induced Granulomatosis Depending on the Infection Route

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 158, No. 9, pp. 303-307, September, 2014
Original article submitted May 19, 2013

Experiments on the model of mouse BCG-induced granulomatous showed that the content of glycosaminoglycans and proteoglycans in the extracellular matrix of the liver and lungs are changed at the early stages of inflammation (days 3 and 30 postinfection) before cell destruction in the organs begins. This is related to degradation of extracellular matrix structures. Their high content in the blood and interstitium probably contributes to the formation of granulomas, fibroblast proliferation and organ fibrosis. These processes depend on the infection route that determines different conditions for generalization of the inflammation process. Intravenous method of vaccine injection is preferable to use when designing the experiments simulating tuberculosis granulomatosis, especially for the analysis of its early stages.

Key Words: *BCG granulomatosis; inoculation route; mice; glycosaminoglycans; hydroxyproline*

The development of tuberculous process is associated with a number of complications including fibrotic ones occurring in all forms of tuberculosis. They often develop in patients with generalized and pulmonary tuberculosis even after successful treatment as the second disease with no effective therapy. For studying of the genesis of fibrotic complications, animal models of tuberculosis are used.

In pulmonary tuberculosis, myofibroblasts are known to synthesize proteoglycan versican and at a sufficient versican levels in the extracellular matrix (ECM) they start to synthesize collagen [6].

It has been previously shown that granulomas are formed in mouse liver and lungs from day 3 to day 30 after BCG infection. However, there are no destructive processes in the granulomas and organs at this term, and hydroxyproline (HOP) levels are increased in both organs [5]. This means that the metabolic changes in the ECM begin much earlier than post-destructive fibrosis develops. So far, little is known about the nature of this process and the participation of other ECM components and their role in early tuberculous granulomatosis.

Being components of ECM in animal organs, proteoglycans and glycosaminoglycans (GAG) similar to collagens are probably involved in the genesis of tuberculous granulomas and fibrosis. In this context, information is needed about changes in these ECM structures at the earliest stages of tuberculous granulomatosis (at the stage of "reception" of the pathogen by phagocytes). The fact that the route of *M. tuberculosis* infection could influence the preferential involvement and the severity of damage to individual organs in tuberculosis makes this problem especially urgent.

Here we studied the levels of sulfated GAG (sGAG) in the liver and lung and HOP as well as total serum GAG in the early stage of BCG-induced granulomatosis and elucidated how the route of *M. tuberculosis* infection affects metabolic parameters of these ECM components in the experiment.

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MATERIALS AND METHODS

Tuberculous inflammation was simulated in 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 mice were maintained on a standard laboratory diet with free access to water and food. The work was conducted in accordance with the Declaration of Helsinki and "Regulation on Animal Experimentation" (Annex to the Order No. 755 of the Ministry of Health, August 12, 1977). The study was approved by the Bioethical Committee of the Research Center of Clinical and Experimental Medicine, Siberian Division of the Russian Academy of Medical Sciences.

BCG vaccine containing *M. tuberculosis* was injected intravenously (into retroorbital sinus) and intraperitoneally in a dose of 0.5 mg/kg body weight in 0.85% NaCl. Each group consisted of 10 animals. Control mice (n=18) received the same amount of 0.85% NaCl. The mice were sacrificed by cervical dislocation under ether anesthesia on days 3 and 30 postinfection (experimental groups) and administration of 0.85% NaCl (control group). After decapitation, the blood was collected for serum, the lungs and liver were isolated; the organs were weighed and frozen at -70°C for further studies.

Proteoglycans were isolated from the liver and lungs [15]. The total content of sulfated and nonsulfated GAG was evaluated in the serum [2] and sGAG levels in the tissues [7]. We used reagents of high purity (Sigma-Aldrich). Optical density of the samples was measured spectrophotometrically on PD-303S (Apel) at the recommended wavelengths. HOP levels in the organs were determined as described previously [5].

The data were statistically processed using Statistica 10.0 software (StatSoft Inc.). The distribution of the values was verified using Kolmogorov–Smirnov test. Mann–Whitney U test was used for the data not conforming normal distribution. Quantitative data are presented as $M \pm m$. The differences between the means were considered significant at $p<0.05$.

RESULTS

Serum. After intraperitoneal injection of BCG, an increase in the content of total GAG relative to the reference level was observed on day 3 and day 30. However, total GAG content on day 30 was lower than on day 3 (Fig. 1). After intravenous injection of BCG, GAG level was higher than in controls (Fig. 1). This parameter was higher on day 30 than on day 3 in the experimental and control groups. After intravenous injection of BCG, GAG levels on day 30 surpassed the corresponding parameter observed at this term after intraperitoneal infection by 3.3 times.

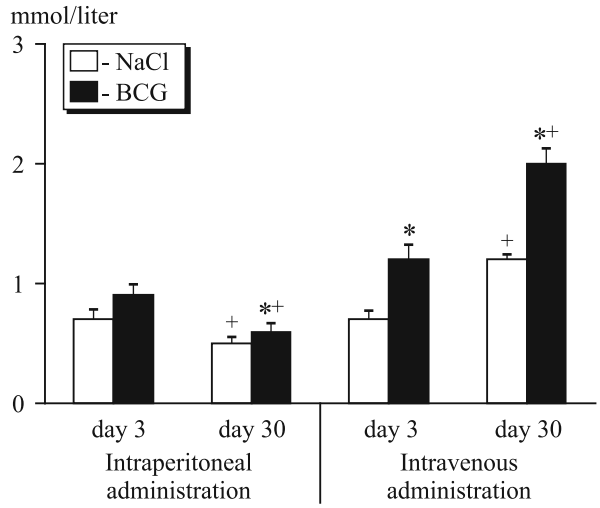

Fig. 1. Serum levels of total GAG depending on the route of BCG inoculation. Here and in Fig. 2, 3: $p<0.05$ in comparison with *corresponding control, +day 3.

Thus, ECM response was evident at early post-infection terms. In this case, the time course of serum content of total GAG varied depending on the route of BCG infection. It was apparently determined by different rates of infection generalization after intraperitoneal and intravenous administration. Since normal GAG levels in the blood are considerably lower than in tissues [11], further studies were aimed at their evaluation in the liver and lungs of infected mice.

Liver. After intraperitoneal BCG administration, sGAG level did not differ from that in the controls on days 3 and 30 (Fig. 2). After intravenous injection of BCG, sGAG level on day 30 was higher than on day 3 and in the control. Thus, intraperitoneal inoculation was not accompanied with significant differences between liver sGAG levels on days 3 and 30, probably due to different rates of generalization of the inflammation process. However, after intravenous BCG administration, sGAG levels increased on day 30 in comparison with those on day 3. As a result, liver sGAG level was 2-fold higher on day 30 after intravenous infection than after intraperitoneal BCG injection at the same term (Fig. 2).

Lungs. After intravenous BCG injection, lung level of sGAG on day 3 did not differ from the control, but was higher than on day 30 (Fig. 3). After intravenous administration, this parameter was higher on day 30 than on post-infection day 3 and in the controls. Thus, after intraperitoneal infection, the level of sGAG in the lungs decreased on day 30; after intravenous infection, it was 4.2-fold higher than after intraperitoneal administration at the same term.

After intravenous BCG injection, the onset and severity of the fibrosis differed in the liver and the

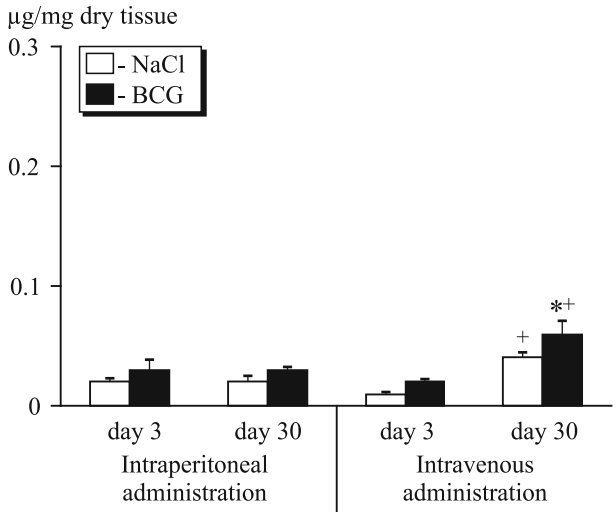

Fig. 2. Liver levels of sGAG in mice depending on the route of BCG inoculation.

lungs [5]. After intraperitoneal inoculation, liver HOP level in mice markedly increased on days 3 and 30 as in comparison with the controls. Lung HOP level was 1.4 times higher on day 30 than on day 3, whereas after intravenous BCG injection, 2.5 times. In this case, the liver did not show the significant increase in this parameter (Table 1).

Proteoglycans and GAG are located in ECM and on the plasma membrane of almost all animal cells; they are among the first host macromolecules that encounter infectious agents [12]. GAG are divided into two groups depending on their structure: sulfated and non-sulfated GAG. The formers, unlike non-sulfated GAG, covalently bind the peptides and form a large variety of proteoglycan. Hyaluronan (non-sulfated GAG) is the major component of ECM and plays a structural role. Furthermore, it can affect a variety of

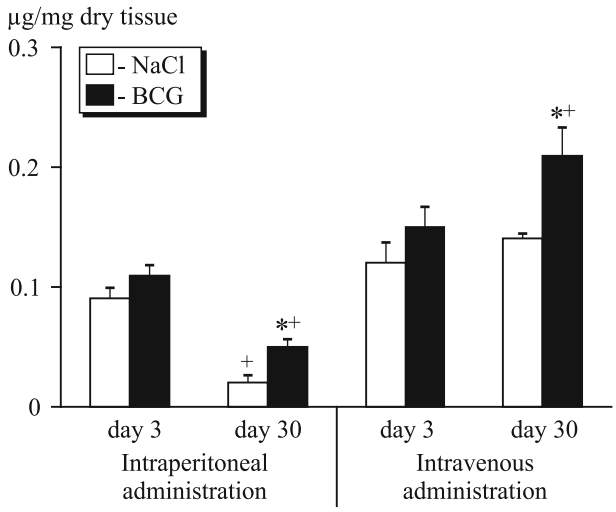

Fig. 3. Lung levels of GAGS in mice depending on the route of BCG inoculation.

cellular functions and processes including inflammation [14]. Involvement of hyaluronan in macrophage aggregation via CD44-receptors during the formation of the primary core of tuberculous granulomas is also discussed [8].

The results of our study showed that GAG levels on post-infection day 30, during the active formation of granulomas in organs, depend on the method of induction of BCG-granulomatosis [5]. GAG level in the serum and in both organs was elevated on day 30 after intravenous inoculation. This may be related to active formation of granulomas and their "maturation" as well as to the fact that GAG are related to the processes of cell proliferation. It was shown that numerical density of granulomas in the lungs and liver increased along with the numerical density of fibroblasts in granulomas at this term after the similar BCG injection in comparison with post-infection day 3.

We cannot exclude another mechanism of GAG increase in tuberculous inflammation associated with increased expression of hyaluronan synthase and accumulation of hyaluronan on the surface of airway epithelial cells, alveoli, and around the granulomas in the lungs of infected mice [10].

Increased GAG levels observed by us can also be associated with disorganization of ECM, altered hydrophilic properties of its components, and their degradation. These changes can be related to the condition of acute stress during early inflammation and enhanced formation of activated oxygen metabolites [3]. During the decay of macromolecular aggregates consisting of hyaluronan and proteoglycans, released hyaluronan can be attacked by *M. tuberculosis* hyaluronidases forming low molecular weight hyaluronan with proinflammatory effect. Interestingly, *M. tuberculosis* exploit host hyaluronan for efficient extracellular replication [10]. The degradation of the proteoglycans including versican, which content increased in pulmonary tuberculosis [6], can increase sGAG levels.

Serum GAG level characterizes the intensity of the proteoglycan metabolism [1], and the level of the circulating hyaluronan is related to the intensity of its release from the peripheral tissues through lymph [11]. The development of the pathological process in a certain organ may affect the serum GAG levels. This may be of diagnostic value in the clinic of internal diseases. Because BCG-induced granulomatosis in this experiment is a generalized process [4] involving many internal organs, altered GAG levels on day 3 and 30 could reflect the catabolic trend of the metabolism of proteoglycans and GAG in the early stage of BCG-induced granulomatosis.

The difference between the liver and lungs in the content of GAG on day 30 depending on the mode of the inoculation can be explained by the structural features of the organism the liver, by the activity of adi-

TABLE 1. Liver and Lung HOP Levels (μg/mg dry tissue) in BALB/c Mice Depending on the Mode of BCG Administration (*M*±*m*)

Organ		Group	
		control (NaCl)	experimental (BCG)
Intraperitoneal administration			
Liver	day 3	1.29±0.09	1.84±0.21*
	day 30	1.99±0.05^{o}	2.85±0.25*^{o}
Lungs	day 3	3.95±0.19	4.38±0.38
	day 30	4.1±0.48	5.82±0.27*^{o}
Intravenous administration			
Liver	day 3	2.44±0.07^{+}	2.81±0.14*$^{+}$
	day 30	2.41±0.12^{+}	2.96±0.06*
Lungs	day 3	5.48±0.47^{+}	7.26±1.05^{+}
	day 30	8.62±0.90^{o+}	18.85±1.13*$^{o+}$

Note. $p<0.05$ in comparison with *corresponding control, ^{o}day 3, $^{+}$intraperitoneal administration.

pocytes, the major ECM producers [9], in the lungs, by surfactant proteins A and D in the alveoli that facilitate the adhesion of mycobacteria to alveolar macrophages and their phagocytosis [13]. The differences in ECM response after intravenous and intraperitoneal administration of mycobacteria found by us are consistent with the concept of the pathogenesis of infectious diseases due to the mode of the inoculation [3].

Thus, ECM changes as a whole after inoculation mice with *M. tuberculosis* at an early stage of inflammation before organ destruction which apparently is associated mostly with the degradation of proteoglycans and GAG. It may facilitate the proliferation of *M. tuberculosis* and also their elimination by phagocytes via endocytosis as well as the proliferation of fibroblasts in the interstitial granulomas. This may contribute to the "fixing" of phagocytic cells in the interstitium at initiation of granuloma formation center (phagocytes become resident cells). All of this is a prerequisite for the development of granulomas, fibroblast proliferation, and organ fibrosis. According to the obtained data, intravenous method of vaccine injection is preferable to use designing the experiments simulating tuberculosis granulomatosis, especially in studying the early stages.

The study was performed using the equipment of the Center for Collective Use "Modern Optical Systems", Research Center of Clinical and Experimental Medicine, Siberian Division of the Russian Academy of Medical Sciences (State Contract No. 16.522.11.7057).

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