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Original research

Crohn's disease patient serum changes protein expression in a human mesenchymal stem cell model in a linear relationship to patients' disease stage and to bone mineral density☆

Martina Blaschke[a,*], Regine Koepp[a], Christof Lenz[c,d], Jochen Kruppa[e,f], Klaus Jung[e,g], Heide Siggelkow[a,b]

[a] Clinic of Gastroenterology and Gastrointestinal Oncology, University Medical Center Göttingen, 37075 Göttingen, Germany
[b] MVZ Endokrinologikum Göttingen, von Siebold-Str. 3, 37075 Göttingen, Germany
[c] Bioanalytical Mass Spectrometry, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany
[d] Institute of Clinical Chemistry, University Medical Center, 37075 Göttingen, Germany
[e] Department of Medical Statistics, University Medical Center Göttingen, Humboldtallee 32, 37073 Göttingen, Germany
[f] Genomics and Bioinformatics of Infectious Diseases, University of Veterinary Medicine Hannover, Institute of animal breeding and Genetics, 30559 Hannover, Germany
[g] Institute of Biometry and Clinical Epidemiology, Charite, University Medical Center Berlin, Chariteplatz 1, 10117 Berlin, Germany

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ABSTRACT

Background: Crohn's disease (CD) is associated with a higher prevalence of osteoporosis, a complication that is recognized as a significant cause of morbidity. Its pathogenesis is controversial, but the activity of CD is one contributing factor.
Methods: We stimulated SCP-1 cells (mesenchymal stem cell line) under osteogenic conditions with serum from adult patients with CD in the symptomatic phase (SP) and in remission (R) and with control sera. Concentrations of IL-6, IL-1 beta, and TNF alpha in the sera were measured. Patients were classified as normal or osteopenic/osteoporotic based on bone mineral density (BMD) T-score measurements. After 14 days in culture, protein expression and gene ontology (GO) annotation analysis was performed.
Results: Cytokine concentrations (IL-6, IL-1 beta, TNF alpha) varied within sera groups. None of the cytokines were significantly increased in the symptomatic phase compared to remission. Protein analysis revealed 17 proteins regulated by the SP versus R phase sera of disease. A linear relationship between CDAI (Crohn's disease activity index) and normalized protein expression of APOA1 and 2, TTR, CDKAL1 and TUBB6 could be determined. Eleven proteins were found to be differentially regulated comparing osteoporosis-positive and osteoporosis-negative sera. Gene annotation and further analysis identified these genes as part of heme and erythrocyte metabolism, as well as involved in hypoxia and in endocytosis. A significant linear relationship between bone mineral density and normalized protein expression could be determined for proteins FABP3 and TTR.
Conclusion: Our explorative results confirm our hypothesis that factors in serum from patients with CD change the protein expression pattern of human immortalized osteoblast like cells. We suggest, that these short time changes indeed influence factors of bone metabolism.

Introduction

Crohn's disease (CD) is a complex multifactorial disease, which is associated with extra intestinal manifestations. One of the organs affected is the skeleton [1]. Therefore, it is important to address the mechanisms of osteoporosis in patients with CD to prevent and treat bone loss [2]. Osteoporotic fractures have a negative impact not only on the quality of life but also on life expectancy [3–5]. CD patients have a 1.2 to 1.7-fold increased relative risk of vertebral and hip fractures, leading to both direct and indirect costs [6–9]. Predicting which individuals have a higher risk remains controversial, yet hypogonadism, less physical activity, malnutrition, malabsorption, vitamin D deficiency or inactivity, disease activity, and glucocorticoid use, in particular, are thought to be the main contributing factors. However, bone

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* Corresponding author.
E-mail address: mblasch1@gwdg.de (M. Blaschke).

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disease may also be present without glucocorticoid treatment [1,10–12,13,14]; therefore, the effect of the disease itself has to be taken into account. Cytokines produced by inflamed intestine in CD may affect bone and hence contribute to bone loss in patients with CD [15]. During the different inflammatory phases in CD cytokines from the gut are secreted into the serum. IL-1 beta, TNF alpha, and especially IL-6 are most likely the main contributing factors to bone deterioration [16]. IL-6 has been identified in sera of children to be responsible for bone loss by decreasing osteoblast differentiation [17]. Interestingly, using serum from children with CD on bone organ cultures only bone formation was decreased and osteoclast activity was unchanged [18]. In newly diagnosed children with CD biochemical bone turnover markers indicated reduced bone formation and also reduced bone resorption [17]. Other studies concerning adult bone have identified IL-1 beta, TNF alpha, and IL-6 to be increased in patients with CD or colitis ulcerosa [15,16,19]. We were able to show with serum from adult patients with CD, that the cytokine combination of IL-1 beta, TNF alpha, and IL-6, but not the individual cytokines of identical concentration, influence bone formation and bone resorption [20]. If bone resorption is induced it seems to be an effect of receptor activator of NF-κB ligand (RANKL) induction, an osteoclast activating soluble ligand produced by osteoblasts and osteocytes, a potent inducer of osteoclastogenesis and activity [15].

Interestingly, not all patients with CD develop osteoporosis. Either the individual bone characteristics or the serum components in these patients may affect or not affect bone health.

We therefore hypothesized here that factors in the serum of patients with CD decisively influence bone turnover. We investigated the effect of serum from patients with CD and healthy individuals on immortalized human osteoblast-like cells. The patient serum was grouped according to different phases of the disease and to bone mineral density (BMD) parameters.

Material and methods

Symptomatic phase and remission sera sampling

We recruited patients with active disease symptoms with CD either at the emergency clinic or at the Clinic for Gastroenterology and Gastrointestinal Oncology (both University Medical Center Goettingen). CD diagnosis was based on endoscopic, histological, or radiological findings. The symptomatic phase of CD was identified using the Crohn's disease activity index (CDAI; supplementary data Table a) [21], in which a score greater than 150 was defined as symptomatic disease. From 23 patients screened during two years only 7 fulfilled the stringent criteria (see below). In another study investigating steroid-free CD patients with active or symptomatic disease, the authors were able to include 99 patients from 34 centers, therefore only around 3 for each center, showing the difficulty of this inclusion criterion [11]. Hence glucocorticoids decisively influence bone metabolism and bone turnover returned to normal in patients not before 4 weeks after cessation of glucocorticoid therapy [22]. Therefore, patients were not included if they received treatment with any steroid or immunosuppressive compound one months prior to analysis in symptomatic disease and in remission. The use of calcium and vitamin D supplements or estrogen was allowed (patient's characteristics please see supplementary data Tables b and c).

Upon inclusion in the study, routine blood analysis was performed. An additional 50 ml blood was drawn, placed directly on ice, and centrifuged within minutes; the resulting serum was batched and stored at −70 °C. When the same patient reached remission and returned for routine examination, another 50 ml blood was drawn, and serum was batched and stored at −70 °C. Steroid treatment had to be finished at least 4 weeks before blood sampling. This study was approved by the Ethics Committee of the University Medical Center Goettingen, and informed consent was signed by all subjects. The characteristics of these patients have been published previously [13]. Briefly, patients were grouped according to their BMD results based on WHO criteria, using a T-score below −1 at the femoral neck or spine to assign patients to the osteopenia/osteoporosis group (Table 1). In addition, sera from healthy individuals (non-CD; non-osteoporotic) were batched and treated like the CD-patient sera.

Table 1
Parameter presenting data (mean values ± standard deviation) comparing osteoporosis positive and osteoporosis negative patients.

Parameter	**Ost+** [c]	**Ost−** [d]	P-value	Total
Sex (male/female)	1/2	2/1		6
T-score femoral neck	**−1.9 ± 0.4**	**0.3 ± 0.97**	**< 0.05**	
T-score spine	**−2.1 ± 0.7**	**0.3 ± 1.15**	**< 0.05**	
IL-6 (pg/mL) *SP*[a]	15.16 ± 10.73	9.8 ± 15.53	ns	12.48 ± 12.3
IL-6 (pg/mL) *R*[b]	8.41 ± 8.03	10.20 ± 15.75	ns	9.3 ± 11.22
P-value	ns	ns		
TNF α (pg/mL) *SP*	5.37 ± 4.66	1.84 ± 3.17	ns	3.61 ± 4.05
TNF α (pg/mL) *R*	56.17 ± 87.33*	6.17 ± 10.68	ns	31.17 ± 62.01
P-value	ns	ns		
IL-1 β (pg/mL) *SP*	0.37 ± 0.078	1.0 ± 1.61	ns	0.68 ± 1.08
IL-1 β (pg/mL) *R*	0.13 ± 0.16	1.34 ± 2.26	ns	0.74 ± 1.58
P-value	ns	ns		
CDAI[e] SP	**349 ± 82**	284 ± 61		**316 ± 73**
CDAI R	**104 ± 71**	91 ± 126		**98 ± 92**
P-value	**< 0.02**	ns		**< 0.001**

Numbers in **bold** are significant values.
[a] SP: symptomatic phase.
[b] R: remission.
[c] Ost+: osteoporosis positive.
[d] Ost−:osteoporosis negative.
[e] CDAI: crohn's disease activity index.
* 1 patient was treated with infliximab in between phases, 3 months prior sampling and showed extremely high TNF alpha values.

Bone densitometry

Bone densitometry using dual X-ray absorptiometry (DXA) was performed at the lumbar spine (L1-L4) and the left femoral neck in all patients with CD during the first week of their stay in the hospital. Osteoporosis was diagnosed when the BMD value was 2.5 or more standard deviations below the mean of a young reference population (T-score), and osteopenia was diagnosed as a T-score below −1. For comparison, the control population data provided by the DXA manufacturer (Hologic, QDR 1000) was used.

Cytokine measurement

Serum cytokine levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's specifications (Table 1). Kits for IL-6 (reference values: 0.447–9.96 pg/ml, minimal detectable level: 0.016–0.110 pg/ml), and IL-1 beta (reference values: < 1.996 pg/ml, minimal detectable level: < 0.1 pg/ml) were purchased from R&D Systems (Minneapolis, USA). For TNF alpha (DPC Biermann GmbH, Bad Nauheim, Germany), the reference values from the manufacturer were < 8.1 pg/ml and minimal detectable level was 0.1 pg/ml. Intra-assay precision was 2.6–3.6%, and inter-assay precision was 4–6.5%.

Cell culture

Disposable products for cell culture were obtained from Nunc (Roskilde, Denmark) and cell culture media and fetal calf serum (FCS)