Is there an autoimmune process in bone? Gene expression studies in diabetic and nondiabetic BB rats as well as BB rat-related and -unrelated rat strains

Nora Klöting,1 Niels Follak,2 and Ingrid Klöting1

Departments of 1Laboratory Animal Science and 2Orthopedic Surgery, Medical Faculty, University of Greifswald, Germany

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Klöting, Nora, Niels Follak, and Ingrid Klöting. Is there an autoimmune process in bone? Gene expression studies in diabetic and nondiabetic BB rats as well as BB rat-related and -unrelated rat strains. Physiol Genomics 24: 59–64, 2005. First published September 27, 2005; doi:10.1152/physiolgenomics.00094.2005.—It is well known that type 1 diabetes is associated with a decrease in bone mass and delayed healing of fractures in human and in animal models of type 1 diabetes. Using well- and poorly compensated diabetic BB/O(titawa) K(arlsburg) rats spontaneously developing insulin-dependent type 1 diabetes, it was recently shown that, in contrast to all other tissues studied, bone is most influenced by metabolic state and seems to be regulated in a manner different from other organs. Therefore, we studied the expression of additional genes (Bmp-1, Bmp-4, Vegf, Bglap, Il-1b, Infg, Tnfa, Calca, Sp1, Yy1) in bone of nondiabetic BB rats compared with newly diagnosed and well- and poorly compensated diabetic rats as well as two nondiabetes-prone congenic BB.SHR rats, BB rat-related (WOKW) and -unrelated rat strains (F344). Six males of each group were euthanized, the tibial bone was removed, and total RNA was extracted, transcribed in complementary DNA, and used for real-time PCR. In a comparison of nondiabetic with diabetic groups, the relative gene expression was reduced by >80% in newly diagnosed and in well-compensated diabetic BB/OK rats. The gene expression in poorly compensated rats increased significantly in 7 of 10 genes and was comparable with those of nondiabetic BB/OK rats. In a comparison of gene expression between diabetes-prone BB/OK and nondiabetes-prone BB.1K, BB.4S, WOKW, and F344 rats, there were no significant differences between newly diagnosed and well-compensated BB/OK diabetic rats and nondiabetic BB.1K, BB.4S, WOKW, and F344 rats. On the basis of these findings, we concluded that spontaneous diabetes influences bone gene expression in BB/OK rats, which may be attributed to the genetically determined autoimmune process not only affecting pancreatic β-cells but also bone formation and resorption.

Type 1 diabetes; congenic rats

IT IS WELL KNOWN that type 1 diabetes is associated with a decrease in bone mass and delayed healing of fractures in human and in animal models of type 1 diabetes (11, 12, 23, 26, 30, 32, 42, 45–48). A number of mechanisms underlying bone abnormalities in diabetes have been proposed. Diminished expression of insulin growth factor-1 (Igf-1), basic fibroblast growth factor (Fgf-1), platelet-derived growth factor (Pdgfb), or affected gene expression may contribute to reduced production of bone matrix; increased brittleness of diabetic bone may be due to abnormalities in microarchitecture (1, 6, 22, 29, 36, 38, 39, 43, 45, 47). With the use of well- and poorly compensated diabetic BioBreeding/Ottawa Karlsburg rats (BB/OK) spontaneously developing insulin-dependent type 1 diabetes at a rate of 86% and a mean age of 103 ± 30 days, it was shown that the metabolic state of rats obviously influenced the extent of delayed bone healing in the early period (2, 8–10, 31). By analysis of the relative gene expression of bone in dependence on metabolic state of BB/OK rats, it was shown that, in contrast to all other tissues studied, bone is most influenced by metabolic state and seems to be regulated in a manner different from other organs studied (14).

These findings prompted us to study the expression of additional genes involved in bone repair and immune regulation per se in nondiabetic BB/OK rats compared with newly diagnosed diabetic and well- and poorly compensated diabetic BB/OK rats to obtain information on the extent to which the diabetic state per se and the metabolic control influence the expression of certain genes. In addition, we analyzed the gene expression of two genetically modified BB/OK rats (congenic BB.SHR rat lines) and one BB rat-related (Wistar Ottawa Karlsburg RT1b, WOKW) and one unrelated rat strain (Fischer rats; F344/Crl) to be able to differentiate between the diabetes-prone and nondiabetes-prone effect on gene expression.

The two congenic BB.SHR rat lines, known as BB.1K and BB.LL or BB.4S, do not spontaneously develop type 1 diabetes because each of them lacks one of the two essential diabetogenic genes, Iddm1 or Iddm2. In congenic BB.1K rats, the diabetes-susceptible major histocompatibility complex (MHC) haplotype RT1b (Iddm1) was replaced by the diabetes-resistant RT1b haplotype of spontaneously hypertensive rats (SHR). Congenic BB.ILL rats are not lymphopenic because the chromosomal region with the lymphopenia gene (Iddm2) was replaced by that of nonlymphopenic SHR rats (15–17, 19).

Like BB/OK, WOKW rats were derived from the same outbred Wistar rat stock of the BioBreeding Laboratories, Ottawa, Canada, more than 20 years ago. In contrast to BB/OK, WOKW rats develop a polygenic and complete metabolic syndrome with obesity, hyperinsulinemia, dyslipidemia, impaired glucose tolerance, hypertension, and proteinuria (18, 20, 21, 44).

Fischer rats (F344/Crl) originated at the Institute for Cancer Research at Columbia University in 1920 and are most probably not related with the founder strain of BB or WOKW (3). We studied the expression of bone morphogenetic proteins (Bmp-1, Bmp-4), vascular endothelial growth factor (Vegf), osteocalcin (bone γ-carboxyglutamic acid protein; Bglap), interleukin-1β (Il-1b), interferon-γ (Infg), tumor necrosis factor-α (Tnfa), calcineurin (Calca), and the transcription factors Sp1 and Yy1. The latter was chosen as internal control to confirm recently published gene expression studies on BB/OK rats (14). Bmp-1 is the prototype of a family of metalloproteases that is capable of inducing formation of cartilage in vivo. In contrast, Bmp-4 belongs to the transforming growth factor-β

Table of Contents

1. Introduction
2. Materials and Methods
3. Results
4. Discussion
5. Conclusion

Address for reprint requests and other correspondence: I. Klöting, Dept. of Laboratory Animal Science, Medical Faculty, Univ. of Greifswald, D-17495 Karlsburg, Germany (e-mail: kloeting@uni-greifswald.de).
superfamily, which influences a broad range of cellular activities, including cartilage development and postnatal bone formation (4, 33). Vegf plays an important role in bone growth via the endochondral ossification pathway and stimulates bone repair by promoting angiogenesis and bone turnover (6). Furthermore, it was shown that there is a synergistic effect on bone healing between angiogenic factor Vegf and osteogenic factor Bmp-4 (38). Bglap is associated with the mineralized matrix of bone and plays an important role in bone healing. Il-1b is a potent stimulator of bone resorption and has been implicated in the pathogenesis of high bone turnover and osteoporosis. Genetic polymorphisms in the Il-1b gene have been reported to be important for bone homeostasis and susceptibility to bone disease (5, 7). Like Il-1b, Infg and Tnfa are proinflammatory cytokines inducing β-cell apoptosis, and the negative effect of both cytokines on osteoclastogenesis has been most extensively characterized (25). We also studied Calca gene expression, which has been found in all cells from yeast to mammals. It couples stimulation of the T-cell antigen receptor to changes in the expression of cytokines and other important immunoregulatory genes. However, Calca not only plays a role in the regulation of the immune response, but it also plays a much broader role in the regulation of cell growth and development. Recently, it was shown that Calca regulates pancreatic growth as well as induces osteoclast differentiation (13, 24). In addition, two transcription factors, Sp1 and Yy1, were selected. Yy1 inhibits Tgfb1 and Bmp-induced cell growth and differentiation, and Sp1 interacts with a variety of gene promoters containing GC-box elements and initiates the transcription of such genes (24).

MATERIALS AND METHODS

Animals. Six nondiabetic male rats, 6 newly diagnosed diabetic BB/OK males (F62), 12 diabetic BB/OK male rats treated with insulin for 4 wk, and 6 males each of congeneric BB.1K (N8F12) and BB.4S (NSF15) as well as of WOKW (F78) and F344 rats (F7F12) were used. Diabetic BB/OK rats were diagnosed as described (30). The age and blood glucose at diabetes onset were 85 ± 6 days and 376 ± 28 mg%, respectively, in newly diagnosed; 83 ± 5 and 389 ± 31 in well; and 85 ± 6 and 358 ± 21 in poorly compensated diabetic rats. The behavior of the two traits after 4 wk of insulin treatment proved to be well-compensated group, as described recently (14).

Blood was obtained by tail vein incision, and blood glucose was measured using a glucose analyzer (ESAT 6660–2, Medingen, Germany). All nondiabetic BB/OK, BB.1K, BB.4S, WOKW, and F344 rats were age matched with diabetic rats treated with insulin for 4 wk (110 ± 5 days). All animals were bred in our own animal facility, kept in Macrolon cages (size 3; Ehret, Emmendingen, Germany) under strict hygienic conditions, and were fed by 10.220.33.3 on May 30, 2017 http://physiolgenomics.physiology.org/ Downloaded from strong major pathogens as described (21). They had free access to food (Ssniff R, Soest, Germany) and water and were maintained in a 12:12-h cycle of light and dark (5 AM/5 PM).

All experiments were performed in accordance with the regulations for animal care of the Ministry of Nutrition, Agriculture and Forestry of the Government of Mecklenburg-Vorpommern (Germany). Animal protocols were approved by the Ministry of Nutrition, Agriculture and Forestry of the Government of Mecklenburg-Vorpommern, Germany.

Insulin treatment. Twelve diabetic animals were treated either with one daily application of 1U insulin (Ultra lente, Novo Nordisk, Denmark) to guarantee survival and to generate a poorly compensated group, or with an insulin implant (osmotic pumps, Charles River Laboratories) that continuously released insulin for 4 wk to obtain a well-compensated group, as described recently (14).

RNA analysis. At the time of euthanasia, tibial bone was removed. The tibial bone was harvested from the proximal metaphysis to the tibiofibular junction, excluding all cartilaginous and soft tissue. The tibias were snap frozen in liquid nitrogen and pulverized. Total RNA was extracted with Trizol (Qiagen, Hilden, Germany). Residual DNA was removed by DNase treatment (RNase-Free DNase Set; Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA concentrations were measured by spectrophotometry. Only RNA samples with an OD260-to-OD280 ratio >1.6 were used for the subsequent experiments. A defined amount of purified RNA (1.5 μg) from all tissue samples was transcribed in cDNA and stored at −20°C until use, as detailed before (14).

Real-time quantitative PCR and expression analysis. Real-time PCR was performed using the ABI PRISM Sequence Detection System 7000 (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions, using ABI PRISM 7000 SDS Software v1.1. as recently described in detail (25). Each quantitative PCR was performed in triplicate. Target cDNAs were amplified, which is produced by primer sets of Bmp-1 (GenBank accession (acc.) no. AB073100; forward (F): 5′-CACACGAGTTGGCCTACGACTA-3′, reverse (R): 5′-CACACGAGTTGGCCTACGACTA-3′), Bmp-4 (GenBank acc. no. NM_012827; F: 5′-GAGGCGCATTCTTCTAGTGTTGTAAT-3′, R: 5′-CTCCACACCATCTTCTGATAATT-3′), Vegf (GenBank acc. no. M25490; F: 5′-ATCGAAGAAAGCCGGCTCACAGT-3′, R: 5′-CTGGCCACCTGTCAGAAGC-3′), Sp1 (GenBank acc. no. AF062644; F: 5′-GGTTCTGGGAAATCTAGACTTCCA-3′, R: 5′-TGTCGACGGTGACGGTGATGGT-3′), Il-1b (GenBank acc. no. NM_031512; F: 5′-CACCCTTCAACGAGAGACACAGA-3′, R: 5′-GGTTCCATGGTGAAGTCAACT-3′), Tnfa (GenBank acc. no. NM_012675; F: 5′-GACAAGGGTCGCCGACTA-3′, R: 5′-AAAGGGCTCTGTAGGCACAGA-3′), Infg (GenBank acc. no. NM_138880; F: 5′-CCGGACCGTGTAGGTCAGG-3′, R: 5′-TGGTTCTGAGGGTACCTCTG-3′), Calca (GenBank acc. no. X57115; F: 5′-GAGGACCGCAGGACGAGAC-3′, R: 5′-GCCAATAGCTCCTGCTCCATC3′), and 18S rRNA (F: 5′-GCCAATAGCTCCTGCTCCATC3′, R: 5′-CCAGCGAGTTGCTACGCTG-3′). The rat 18S rRNA gene (eukaryotic 18S rRNA endogenous control; FAM Dye/MGB Probe, Applied Biosystems) served as the endogenous reference gene. The melting curve was done to ensure specific amplification.

The standard-curve method was used for relative quantification. For each experimental sample, the amounts of targets and endogenous reference, 18S rRNA, were determined from the calibration curve. The target amount was then divided by the endogenous reference amount to obtain a normalized target value.

Statistical analysis. Results are expressed as means ± SD. Differences were assessed by one-way analysis of variance corrected by Bonferroni-Holm using the Statistical Package for the Social Sciences (SPSS, Chicago, IL).

RESULTS

As shown in Fig. 1, the relative gene expression was measured in Tnfa, ranging from 500 to 5,000, followed by Bglap, Infg, Bmp-2, and Il-1b, ranging from 40 to 1,000, and by Sp1, Yy1, Calca, Bmp-1, and Vegf, ranging from 2 to 60 in all animal groups studied. In a comparison of nondiabetic with diabetic groups, the relative gene expression was reduced by >80% in newly diagnosed and in well-compensated diabetic BB/OK rats 4 wk after insulin treatment. There were no significant differences between newly diagnosed and well-compensated diabetic, but there were significant differences in gene expression in
9 of 10 genes studied between newly diagnosed and poorly compensated rats, and in 3 (Vegf, Bmp-1, Il-1b) of 10 genes between well- and poorly compensated rats. Comparable behavior in gene expression of all genes studied was observed for Bmp-1 and Il-1b, Calca, Sp1, and Bmp-4 among all groups.

In a comparison of gene expression between nondiabetic and newly diagnosed diabetic BB/OK rats and their genetically modified derivatives, BB.1K and BB.4S, as well as WOKW and F344 rats (as summarized in Fig. 2), it is obvious that nondiabetic BB/OK animals showed the highest expression of all rats studied. In contrast, newly diagnosed diabetic rats showed an expression that was comparable with all nondiabetic rat strains. There were no significant differences between newly diagnosed BB/OK diabetic and nondiabetic BB.1K, BB.4S, WOKW, and F344 rats.

**DISCUSSION**

It was clearly demonstrated that diabetes onset decreases the relative expression of all genes studied in bone by >80%, and that good compensation of diabetes in BB/OK rats did not significantly increase the expression of genes compared with newly diagnosed diabetics. In contrast, the poorly compensated diabetics showed a significant increase of expression in 7 of 10 genes. The expression was comparable with those of nondiabetic BB/OK rats. However, there were three exceptions. The expression of Vegf, Yy1, and Bglap was significantly decreased in poorly compensated rats compared with nondiabetics. These findings clearly demonstrate that the expression of most genes was independent of metabolic control in bone, confirming our earlier findings (14).

Studies on gene expression of osteocalcin, collagen type I, and transcription factors in mice with streptozotocin-induced diabetes clearly showed that the gene expression was substantially reduced in diabetic mice compared with nondiabetic controls, but insulin treatment of these diabetic mice significantly increased the expression of genes studied (osteocalcin, collagen type I, transcription factors Dlx5, and Cbfal/Runx-2) (29). Regarding the metabolic state, the untreated diabetic mice may be comparable with our poorly compensated rats. Because newly diagnosed and well-compensated diabetic rats were characterized by obviously lower expression in all genes compared with nondiabetic BB/OK, the inconsistent findings between diabetic mice and BB/OK rats may be explained by the fact that the bones of animals with streptozotocin-induced diabetes and not of spontaneously diabetic animals were studied. Streptozotocin is a drug that selectively destroys insulin-producing β-cells of the pancreas, leading to insulin-dependent type 1 diabetes. In contrast, type 1 diabetes in spontaneously diabetic BB rats is caused by a genetically determined autoimmune process by which β-cells are selectively destroyed. It seems that gene expression in bone of spontaneously diabetic BB/OK rats is regulated differently from artificially induced diabetes. Considering this surprising finding that the gene expression was obviously reduced in newly diagnosed and well-compensated diabetics compared with nondiabetic BB/OK rats, we hypothesized that the
genetically determined autoimmune process took place not only in the pancreas, leading to β-cell destruction, but also in bone, influencing bone formation and resorption. If so, genetically modified BB/OK rats in which the autoimmune process does not lead to type 1 diabetes should give an answer.

Therefore, we studied two congenic BB.SHR rat lines known as BB.1K and BB.LL or BB.4S. Rats of both congenic rat strains do not spontaneously develop type 1 diabetes, because each of them lacks one of the essential diabetogenic genes (15-17, 19). The findings clearly demonstrated that the gene expression of all genes studied was significantly reduced in both congenic BB.SHR lines compared with nondiabetic BB/OK rats. Therefore, the gene expression in nondiabetes-prone BB.1K, BB.LL, WOKW, and F344 rats was comparable with those of newly diagnosed and well-compensated diabetic BB/OK rats and significantly reduced compared with nondiabetic BB/OK rats.

These findings may serve as indirect evidence that the autoimmune process in prediabetic, normoglycemic BB/OK rats affects not only β-cells but also bone, as indicated by increased gene expression. The autoimmune process is more or less finished when most β-cells are destroyed and hyperglycemia is manifested, which may be followed by “normalization” of gene expression in bone of diabetic BB/OK rats; therefore, the gene expression is comparable with those of nondiabetes-prone BB.1K, BB.LL/BB.4S, WOKW, and F344 rats.

There are several studies in bone clearly demonstrating that the skeletal and immune systems regulate each other to a much greater degree than previously believed. In particular, various pathological conditions that lead to excessive bone loss, such as rheumatoid arthritis, periodontal disease, and some tumor-associated bone abnormalities, have been shown to be influenced by cellular components, e.g., T lymphocytes, as well as by soluble factors produced by infiltrating lymphocytes, e.g., \textit{Infg} (35). As in type 1 diabetes development, activated T cells also play a crucial role in osteoclastogenesis. Among the inflammatory cytokines, the negative effect of \textit{Infg} on osteoclastogenesis has been most extensively characterized. Mice injected with \textit{Infg} show defects in the formation of multinucleated osteoclasts, and \textit{Infg} produced by T cells blocks osteoclastogenesis (35, 40). \textit{Tnfa} and other members of the \textit{TNF} family not only regulate physiological bone remodeling, but they are also implicated in the pathogenesis of various bone

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Fig. 2. Relative gene expression (mean ± SD) in bone of nondiabetic (nd) and newly diagnosed diabetic BB/OK (d), BB.1K, BB.4S, WOKW, and F344 rats. ***Significant differences between nondiabetic BB/OK and newly diagnosed BB/OK, nondiabetic BB.1K, BB.LL, WOKW, and F344 rats (P < 0.001).
diseases such as osteoporosis and bone loss in inflammatory conditions. In addition, the meaning of *II-1b* as a potent stimulator of bone resorption and its implication in the pathogenesis of high bone turnover and osteoporosis is well documented. These cytokines are also implicated as a pathogenic factor in initial events of β-cell destruction leading to type 1 diabetes. Destructive insulitis is associated with increased expression of *II-1b*, *Tnfα*, and *Infγ* (34). Therefore, there are parallels between the same cytokines implicated in β-cell destruction and in osteoporosis and bone loss. That could mean that similar or even the same processes take place in both β-cells and bone of spontaneously diabetes-prone BB rats. Although this would be a new idea, supporting evidence has already been found in humans (28).

A high percentage of patients with type 1 diabetes have osteopenia at the time of clinical diagnosis. This indicates the existence of pathogenic mechanisms operating before the overt manifestation of type 1 diabetes (28). It is well established that β-cell destruction and insulinitis begin several years before the onset of clinical recognition of disease. Therefore, our assumption could be that some autoimmune and autoinflammatory responses, ongoing before diabetes onset, may also affect bone and play a role in bone loss (37). This idea is also supported by recently published findings measuring bone mineral density (BMD), diabetes, and bone-relevant traits in teenage-age (13–19 yr) and postteenage females (20–37 yr) with type 1 diabetes compared with appropriate age-matched control subjects. Results of this study showed that women with type 1 diabetes exhibit BMD differences early in life, with significant differences already present in postteenage years. In addition, there was no association between BMD measurements and metabolic control or diabetes duration (27).

In conclusion, the present study clearly demonstrates the influence of spontaneous diabetes on bone gene expression in BB/OK rats, which may be attributed to the genetically determined autoimmune process that not only affects pancreatic β-cells, but may also affect bone formation and resorption.

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