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# Intact growth factors are conserved in the extracellular matrix of ancient human bone and teeth: a storehouse for the study of human evolution in health and disease

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## Abstract

For the first time we have extracted, solubilized and identified growth factors, such as insulin growth factor II (IGF-II), bone morphogenetic protein-2 (BMP-2), and transforming growth factor-β (TGF-β), from archaeological compact human bone and tooth dentin dating from the late pre-ceramic pottery Neolithic (late PPNB) and the early Middle Ages. These factors are typical of special physiological or pathological situations in the metabolism of bone. The extracellular matrix proteins from bone and teeth of individuals from the late PPNB and early Middle Ages were separated by 2-D electrophoresis and more than 300 different protein spots were detected by silver staining. The matrix protein patterns of compact bone and tooth from the same individual (early Middle Ages) are very different and only 16% of the protein spots were detected in both compact bone and tooth dentin.

**Keywords:** ancient bone and teeth; bone morphogenetic protein-2 (BMP-2); 2-D electrophoresis; human evolution; insulin growth factor II (IGF-II); transforming growth factor-β (TGF-β).

## Introduction

Bone is a highly dynamic tissue that has evolved over millions of years under gravitational stress on Earth to provide mechanical support for both locomotion and protection, to serve as a calcium reservoir for mineral homeostasis, and to support hemopoiesis (Einhorn, 1996). In the last two decades, increasing numbers of extracellular matrix (ECM) proteins have been detected that are produced locally in bone or are trapped within the hard tissue matrix and play a critical role in regulating normal and pathological skeletal growth and remodeling.

For some non-collagenous matrix proteins, several aspects relating to function are becoming clearer. For example, osteopontin has a Gly-Arg-Gly-Asp-Ser (GRGDS) amino-acid sequence that promotes osteoclast attachment via cellular integrin $\alpha_v\beta_3$ (Reinholt et al., 1990; Heinegard et al., 1995). Bone alkaline phosphatase (BALP) is a glycoprotein that functions as an ectoenzyme attached to the osteoblast cell membrane by a glycosyl phosphatidylinositol (GPI) anchor (Fedde et al., 1988; Hooper, 1997). BALP has been reported to be necessary for the initiation of mineralization by osteoblast-derived vesicles, but not for continuation of the process (Tenenbaum, 1987; Bellows et al., 1991; Barling et al., 1999). SPARC (osteonectin) has been shown to be a $Ca^{2+}$-binding glycoprotein that functions as a counteradhesive protein, as a modulator of growth factor activity, and as a cell-cycle inhibitor. In adults, the expression of osteonectin is limited largely to tissues undergoing repair or remodeling due to wound healing. However, pathological processes, such as cancer metastasis, arthritis, diabetes and kidney diseases, are also characterized by elevated expression of SPARC (Reed and Sage, 1996). It seems to be expressed in the very early stages of cell differentiation or by osteoblast progenitor cells and it may maintain an environment for sequential maturation of osteoblasts by its protein accumulation and matrix formation with other gene products (Ibaraki et al., 1992).

Bone and teeth are the hardest tissues in vertebrates. After death, a corpse that has not been embalmed leaves only bone and teeth, as a rule, as remnants that can be found after hundreds or thousands of years. Of course, the protective effect of the soil covering a corpse plays an important role in the preservation of such material. Thus, bony tissues are preserved in highly different ways and to different degrees.

Our most reliable knowledge about human evolution is established by morphological examinations of bone and teeth using macroscopic and microscopic techniques. In recent years, some molecular information on ancient bone and teeth has been obtained from extracted short DNA chains (approximately 200 or 300 bp; Krings et al., 1997). It is now possible to gather molecular information from intact ECM proteins of bone and teeth from subfossil and fossil specimens. The turnover in compact (cortical) bone per year is 4%, whereas in trabecular bone the turnover is 28% per year (Parfitt, 1994). Tooth dentin is a convenient material for the investigation of age-related modifications of proteins and certain studies have indicated that this reaction is primarily restricted to non-collagenous proteins (Masters, 1985; Kuboki et al., 1984).

We have already identified osteopontin, osteonectin, osteocalcin, alkaline phosphatase and bone sialoprotein in fresh, macerated bones and in ancient bones (Schmidt-Schultz and Schultz, 2004, 2005). It is remarkable that it is also possible to extract and identify intact ECM proteins from ancient bones because of the more efficient solubilization process inherent in this technique (Schmidt-Schultz and Schultz, 2004). Therefore, protein

separation has been improved and there is a better guarantee of identification by specific antibodies.

Bone has a remarkable regenerative potential that enables it to remodel itself in response to changing physical demands and to repair itself after injury. Important for the process of remodeling and self-repair are growth factors. Several growth factors, such as transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs) and insulin growth factors (IGFs) have clear functions in the regulation of bone formation during the healing process, for example, after fracture (Andrew et al., 1993; Mundy 1993; Taniguchi et al., 2003).

BMPs are members of a class of ancient highly conserved signaling molecules that play major roles in embryonic axis determination, organ development, tissue repair, and regeneration throughout the animal kingdom (Kaplan and Shore, 1998). BMPs were discovered because of their ability to induce cartilage and bone formation from non-skeletal mesodermal cells and are now of considerable interest as therapeutic agents for healing fractures and periodontal bone defects (Urist, 1994; Niederwanger and Urist, 1996).

IGFs stimulate osteoblast cell proliferation and differentiated functions, such as type I collagen expression (Canalis et al., 1993). Furthermore, besides its mitogenic effects, the IGF peptide increases the production of several bone matrix proteins, stimulates collagen type I expression, and inhibits collagen degradation, possibly through down-regulation of collagenase expression, and induces alkaline phosphate expression (Langdahl et al., 1998).

TGF-β has been observed to both inhibit and stimulate osteoblast cell proliferation, in part depending on TGF-β concentration, species and stage of osteoblast differentiation (Centrella et al., 1994). The amount of TGF-β in human bone declines with age (Nicolas et al., 1994). It is proposed that latent TGF-β in the bone cell microenvironment is activated by acidic conditions, which develop beneath the ruffled border of active osteoclasts (Centrella et al., 1994), and by proteolytic compounds of the plasminogen system (Yee et al., 1993). Similar to the IGFs, TGF-β expression in periosteal tissue rapidly increases with external mechanical loading (Raab-Cullen et al., 1994).

Many molecules, whether produced locally or exogenously, are adsorbed on to bone hydroxyapatite during life. Mineralizing bone provides an excellent system for the sequestration and subsequent immobilization of molecules normally soluble in physiological fluid.

This junkyard-like property of bone can be quite advantageous if the sequestered molecules afford benefits to the tissue, either in its normal physiology or under stress. The sequestered proteins, which are entrapped in the ECM of bone during its lifetime, can be solubilized using the method described by Schmidt-Schultz and Schultz (2004) not only for recent bone, but also for bones that are thousand of years old. Thus, we have extracted, solubilized and identified growth factors, such as IGF-II, BMP-2, and TGF-β, conserved in archaeological human bone and teeth that are typical of special physiological or pathological situations in the metabolism of bone and teeth, dating from the late pre-ceramic pottery Neolithic (late PPNB) and early Middle Ages. The identification of ECM proteins in ancient bones and teeth is an important approach in the field of molecular paleopathology. This is a new field of research in which molecules are used to confirm the presence of disease in past populations. It is this field that will contribute to our study of the history of diseases, their frequency over the course of time, and the evolution of disease.

A precondition for biochemical analysis is that archaeological bone samples are primarily controlled by microscopic techniques (histology) to exclude changes due to postmortem processes (diagenesis), which might damage the microstructure of the original bone substance significantly and might also lead to false biochemical results (Schultz, 1997; Schmidt-Schultz and Schultz, 2004). Thus, the first step in identifying proteins in recent and especially in archaeological bone samples is to establish the preservation state of organic and inorganic bone structures. This is carried out microscopically by viewing thin ground sections in plane and polarized light. In this way, contamination and destruction caused by decomposition (destruction of organic structures) and diagenesis (destruction of inorganic structures) in ancient bone can be detected and ruled out (Schultz, 1986, 1997; Hansen and Buikstra, 1987; Schoeninger et al., 1989). If there is no contamination and destruction due to plant roots, fungi, algae, bacteria, arthropods, etc., the sample is suitable for protein extraction (Figure 1).

The aim of this study is to demonstrate the extraction, solubilization and identification of growth factors that are typical of special physiological or pathological situations in bone metabolism from archaeological human skeletal remains. Furthermore, for the first time, differences in the ECM protein patterns of human bone and tooth dentin from the same individual are demonstrated by two-dimensional electrophoresis.

## Results

### Protein extraction and separation of bone and tooth proteins

ECM proteins were extracted from samples of human compact (cortical) bone substance and tooth dentin taken from a recent individual and from archaeological specimens dating from the late PPNB and the early Middle Ages (Table 1). These samples and recent human bone and tooth dentin used as controls were examined using silver-stained 1-D-electrophoresis (SDS-PAGE). A clear protein pattern of approximately 25–30 colored bands in the molecular mass range of about 10–150 kDa was observed (the detection limit for silver is in the range 1–10 ng of protein). Figure 2 (lane 1) shows ECM proteins of the compact bone substance of a recent adult individual. In lane 2, the protein pattern of dentin (tooth 31) of a recent juvenile individual is presented. Lane 3 (compact bone substance) and lane 4 (dentin of tooth 13) display ECM proteins of an adult individual from Barbing (early Middle Ages, 450–600 AD). The proteins shown in lane 5 (compact bone substance) and lane 6 (dentin of tooth 27) were extracted from an adult individual from Basta, a late PPNB site in Jordan (7500–6000 BC). It is striking

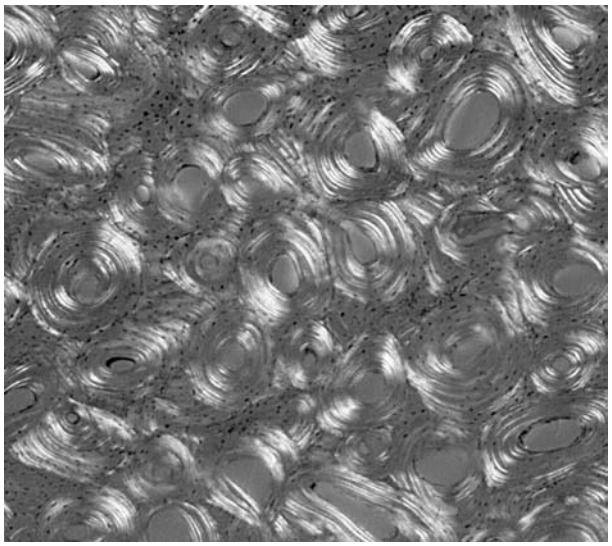

**Figure 1** Microstructure of well-preserved ancient compact bone substance (early Middle Ages).
Thin ground section (50 μm) viewed in polarized light. Photomicrograph taken with 350× magnification.

that ECM proteins could be extracted, solubilized and identified from samples that were almost 9000 years old. Lane 7 shows a molecular mass marker.

### Western blot analysis

Some of the typical growth factors of bone were identified in recent and ancient compact bone and tooth dentin by Western blotting using specific antibodies (Figure 3A; Western blot with antibodies against human TGF-β, a growth factor of approx. 25 kDa). ECM of human compact bone taken from a recent individual (lane 1), from an individual from the early Middle Ages (lane 2), and an individual from the late PPNB (lane 3) clearly shows strong bands in the region of 25 kDa. In the ECM of human tooth dentin from a recent individual (lane 4) and from human archaeological specimens from the early Middle Ages (lane 5) and the late PPNB (lane 6), the presence of TGF-β is also clearly evident. However, the intensity of TGF-β in tooth dentin is apparently weaker than in compact bone substance.

Figure 3B shows Western blotting of ECM proteins from human compact bone and dentin with antibodies against human BMP-2. In all six samples, we detected the presence of human BMP-2 (compact bone in: lane 1, recent individual; lane 2 individual from the early Middle Ages; lane 3, individual from the late PPNB; tooth dentin: lane 4, recent individual; lane 5, individual from the early Middle Ages; lane 6, individual from the late PPNB). ECM proteins from compact bone were controlled by a blocking peptide against human BMP-2. The typical bands in the region of ca. 25 kDa representing human BMP-2 were totally blocked (lanes 8–10; lane 7 shows the molecular mass marker).

For the third growth factor, human IGF-II, we examined the same samples of human compact bone and tooth dentin as for TGF-β and BMP-2 (Figure 3C). Human IGF-II is clearly present in five of the six samples (compact bone in: lane 1, recent individual; lane 2, individual from the early Middle Ages; lane 3, individual from the late PPNB; tooth dentin: lane 4, recent individual; lane 5, individual from the early Middle Ages; lane 6, individual from the late PPNB). Compact bone from the individual dating from the late PPNB shows only a very faint band in the region of 7.5 kDa, whereas tooth dentin from the same individual shows a clear band of IGF-II.

In the three samples of tooth dentin, the ECM proteins were investigated using a blocking peptide against human IGF-II. In Figure 3C, lanes 8–10 show the typical position at ca. 7.5 kDa for IGF-II that was totally blocked. Lane 7 shows the molecular mass marker.

These results demonstrate that the presence of growth factors can also be demonstrated in compact bone and tooth dentin taken from individuals excavated from archaeological sites. Although these factors have an enormous influence on modeling and remodeling of bone in health and disease, the activated form of these growth factors is only found at a relatively low concentration in bone.

### 2-D electrophoresis

1-D electrophoresis resolves bone and tooth dentin ECM proteins according to their molecular weight. The protein mixture contains many proteins with the same molecular mass. This means that in the same band in a 1-D SDS-

**Table 1** Bone and tooth samples.

| # | Bone | Sex | Age (years) | Preservation | Source |
|---|---|---|---|---|---|
| Recent bones | | | | | |
| 1 | Right femur | Male | 38 | Frozen directly after dissection | Dept. Pathology/Anatomy, University of Göttingen |
| 2 | Tooth 31 | Male | Juvenile | Frozen after dissection | Dental Medicine, University of Tübingen |
| | Bone | Sex | Age | Archaeological site | Chronology |
| Ancient bones | | | | | |
| 3 | Right femur | Female | 21–25 | Barbing, Germany | 450–700 AD |
| 4 | Tooth 13 | Female | 21–25 | Barbing, Germany | 450–700 AD |
| 5 | Left femur | Male | 30–39 (45) | Basta, Jordan | 7500–6000 BC |
| 6 | Tooth 27 | Male | 30–39 (45) | Basta, Jordan | 7500–6000 BC |

Bone and tooth dentin from the same individuals dating from recent times, from early middle ages and from the earliest Neolithic (late PPNB) were examined. As controls, bone and tooth dentin of recent times were used for this study.

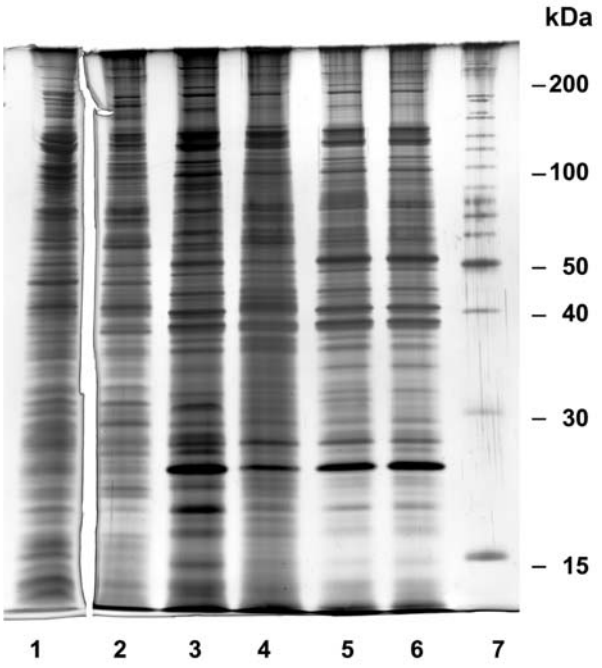


**Figure 2** 1-D electrophoresis of human ECM proteins from compact bone and tooth dentin stained with silver.
Lane 1, recent compact bone; lane 2, recent tooth (31) dentin; lane 3, compact bone, early Middle Ages; lane 4, dentin of tooth 13, early Middle Ages; lane 5, compact bone, late PPNB; lane 6, dentin of tooth 27, late PPNB; lane 7, molecular mass marker.

PAGE gel, several proteins are concentrated at the same place. To calculate the quantity of proteins present in the bone ECM and, in particular, in the same band of a 1-D electrophoresis gel, it is necessary to separate the proteins by 2-D electrophoresis. In the first dimension, the proteins are resolved according to their isoelectric point. In the second dimension, they are resolved according to their molecular weight in SDS-PAGE.

In Figure 4 we present 2-D electropherograms of the ECM proteins from recent compact bone (Figure 4A) and recent dentin of tooth 31 (Figure 4B). Results for the archaeological specimens are shown for the individual from the early Middle Ages in Figure 5A (compact bone) and Figure 5B (dentin of tooth 13), and for the individual from the late PPNB in Figure 6A (compact bone) and Figure 6B (dentin of tooth 27). It is interesting that the protein patterns for the recent and archaeological samples of compact bone are more or less similar, whereas they greatly differ from the patterns for tooth dentin samples, which are similar to each other.

We compared the ECM proteins obtained from compact bone and dentin of tooth 13 for the individual from the early Middle Ages using the software PDQuest 2-D 7.0 (Bio-Rad, Munich, Germany). The results of the matching process are interesting. Only 16% of protein spots present in the tooth dentin were also found in the compact bone substance (Table 2). In Figure 7A,B, protein spots identified letters are found in bone and tooth dentin, whereas the spots marked by circles can only be found in bone or in tooth dentin. Every protein spot stained by silver represents a different matrix protein. We counted the protein spots for compact bone and tooth dentin from the individual from the Middle Ages using PDQuest 2-D 7.0. The total spot count was 338 in the compact bone sample and 335 in the tooth dentin sample from the same individual. Boskey (1992) assumed that approximately 200 different matrix proteins would be present in bone. Also, if we consider that several of the extracted matrix proteins represent isoenzymes, we can very probably solubilize most, if not all, extracellular matrix proteins present in compact bone and tooth dentin.

## Discussion

A key aspect of this advancement is the ability to resolve the individual components of a complex mixture of ECM proteins of compact bone and tooth dentin from recent and archaeological individuals before analysis. In contrast to other organic materials, bone tissue has higher probability of being preserved over a long time span due to its physical durability and resistance against various factors, for instance, decomposition and diagenesis (Schultz, 1997; Schmidt-Schultz and Schultz, 2004). Cell

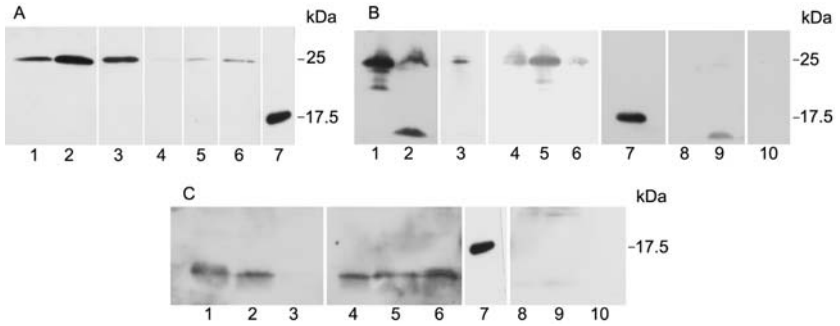


**Figure 3** Western blot for different growth factors in human compact bone and tooth dentin from different time periods.
(A) Antibodies against human TGF-β: lanes 1–3, compact bone of recent times, early Middle Ages and late PPNB; lanes 4–6, dentin of tooth 31, recent times, dentin of tooth 13, early Middle Ages, dentin of tooth 27, late PPNB; lane 7, molecular mass marker. (B) Antibodies against human BMP-2: lanes 1–3, compact bone of recent times, early Middle Ages, late PPNB; lanes 4–6, dentin of tooth 31, recent times, dentin of tooth 13, early Middle Ages, dentin of tooth 27, late PPNB; lane 7, molecular mass marker; lanes 8–10, BMP-2 bands blocked with special blocking peptides in compact bones of recent times, early Middle Ages and late PPNB. (C) Antibodies against human IGF-II: lanes 1–3, compact bone recent times, early Middle Ages, late PPNB; lanes 4–6, dentin of tooth 31, recent times, early Middle Ages, late PPNB; lane 7, molecular mass marker; lanes 8–10, IGF-II bands blocked with special blocking peptides in tooth dentin from recent times, early Middle Ages, and late PPNB, respectively.

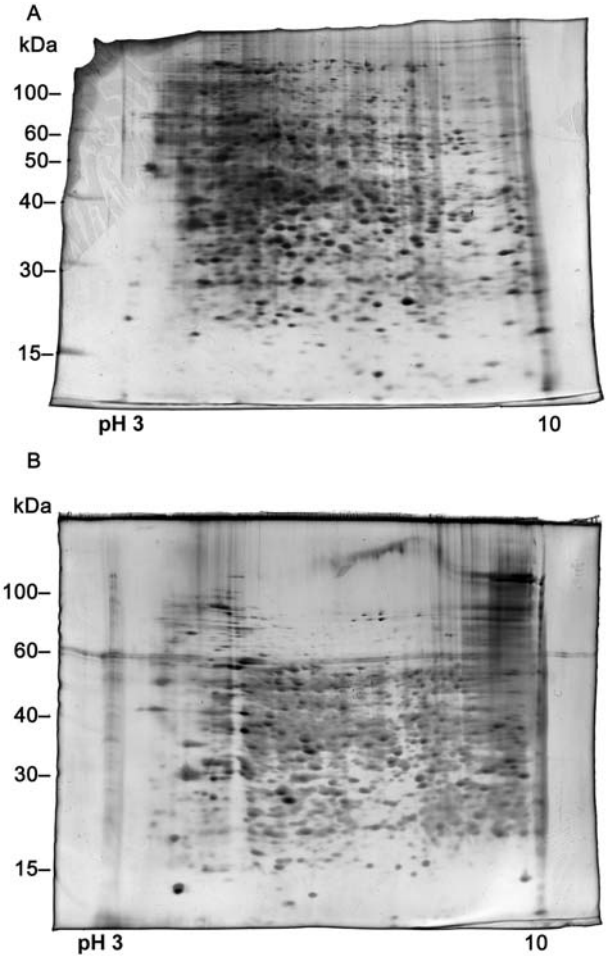


**Figure 4** 2-D electrophoretic analysis of recent specimens.
First dimension, IPG-Strip pH 3–10; second dimension, SDS-PAGE (12% T, 2.5% C) stained with silver; left-hand side, molecular mass marker. (A) ECM proteins from recent compact bone; (B) ECM proteins from recent dentin of tooth 31.

proteins are rapidly broken down after death by autolytic processes. The proteins of the ECM of bone and tooth are much better protected. The apatite in which the proteins are embedded apparently provides considerable protection against the postmortem destruction caused by chemical and physical agents. Thus, these structures might be preserved for many thousands of years after death. Frequently, ECM proteins of bone are conserved in ancient bones and teeth, as well as in fresh tissues. Before the extraction of proteins from recent and ancient bone and teeth samples was carried out, the preservation state of the organic and inorganic bone structure was investigated microscopically by viewing thin ground sections in plane and polarized light. During recent years, we have applied our extraction protocol to many different bone samples from various populations, located in different geographical areas and dating from different time periods (Schmidt-Schultz and Schultz, 2004). We have demonstrated that it is possible to extract intact ECM proteins from ancient bone tissue. This has been verified by checking and rechecking by repeated extractions and repeated experiments whether the ancient bone is in a good preservation state or not, with comparable results.

Using reliable techniques, it is possible to identify these proteins, for example collagen type I, osteonectin, osteopontin, osteocalcin, alkaline phosphatase, bone sialoprotein and the main antibody of the immune system, IgG (Schmidt-Schultz and Schultz, 2004, 2005). The field of bone ECM proteomics applied to archaeological skeletal remains dating not only from hundreds and thousands, but even hundreds of thousands of years, provides a tremendous opportunity for further research into human evolution. The dramatic environmental, cultural and behavioral changes in human societies during the past 10 millennia have been key determinants of human disease. The shift from small nomadic populations to larger settled populations and the parallel changes in diet from a hunter-gatherer-based subsistence to the beginnings of settled populations who developed agriculture, affected both the plants and animals that were being domesticated and the human populations. Man began to domesticate animals as pets and livestock, to cultivate plants for food and to dramatically transform his environment. It may not have happened all at once, nor were the events necessarily dependent upon each other; however, the changes in the make-up of these early societies and the altered diets each had dramatic

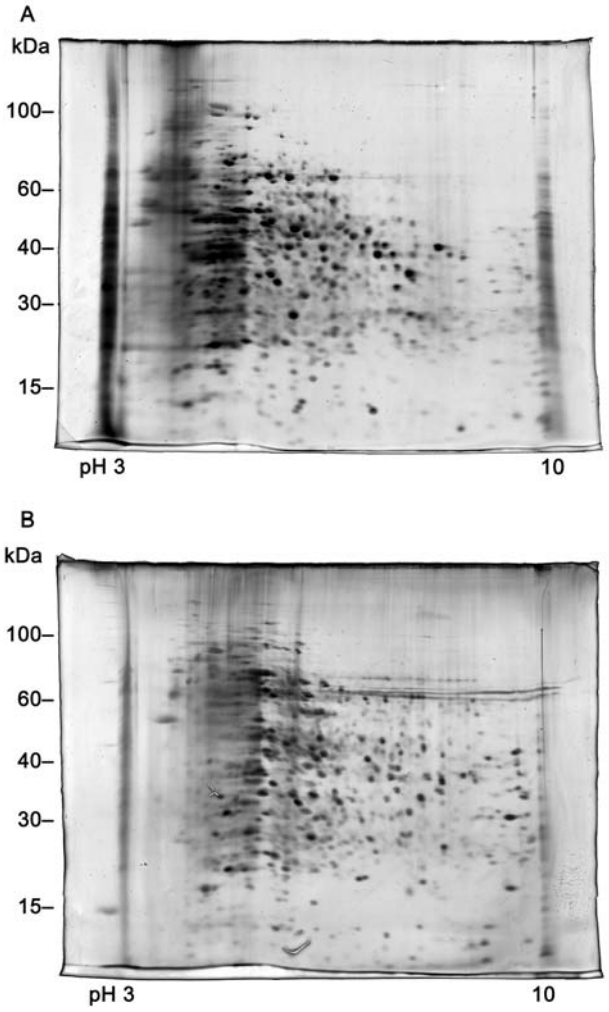


**Figure 5** 2-D electrophoretic analysis of specimens from the early Middle Ages.
First dimension, IPG-Strip pH 3–10; second dimension, SDS-PAGE (12% T, 2.5% C) stained with silver; left-hand side, molecular mass marker. (A) ECM proteins from compact bone, early Middle Ages; (B) ECM proteins from dentin of tooth 13, early Middle Ages.

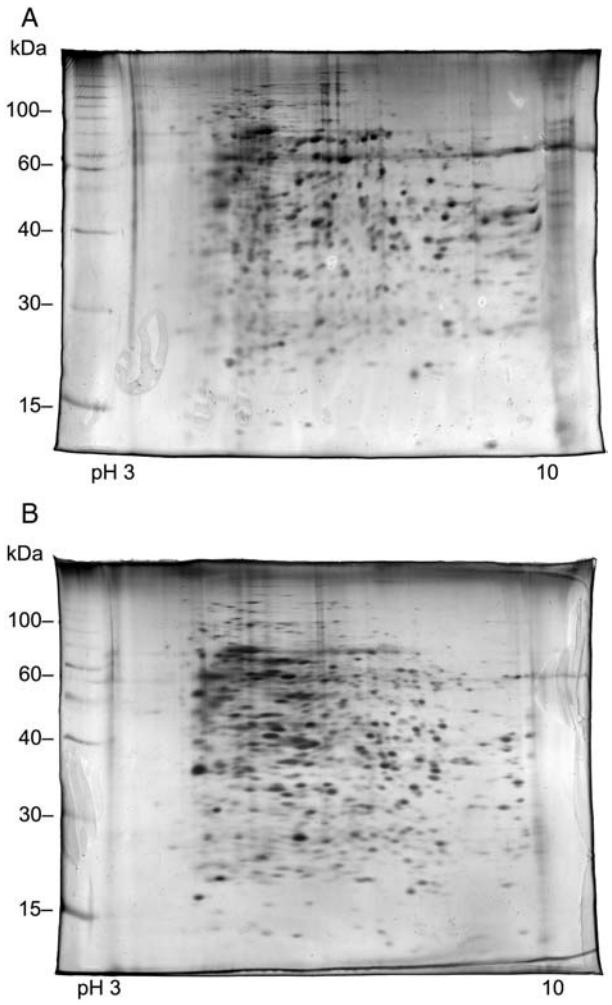


**Figure 6** 2-D electrophoretic analysis of specimens from the late PPNB.
First dimension, IPG-Strip pH 3–10; second dimension, SDS-PAGE (12% T, 2.5% C), stained with silver; left-hand side, molecular mass marker. (A) ECM proteins from compact bone, late PPNB; (B) ECM proteins from dentin of tooth 27, late PPNB.

effects on the type and diversity of human diseases (Diamond, 1997). Upon exposure to a pathogen, the principle human defense is the immune system, which generates a huge diversity of antibodies and T-cell receptors, through a complex process of genetic recombination and splicing, with which to target a potentially infinite number of pathogens.

The most abundant antibody in human tissues, IgG, was identified with antibodies against the heavy chains in the bones of five prehistoric children (Schmidt-Schultz and Schultz, 2004). The protein contents in the Western blots of these children were comparable and were determined quantitatively by a modified Lowry procedure (Bensadoun and Weinstein, 1975). One of these children, a 14–16-year-old child from the bronze age in Franzhausen, Austria (2200–1800 BC) suffered from scurvy and had the lowest IgG content. It is well known that chronic scurvy produces subperiosteal bleeding. The bone tissue can be changed by an organization process (healing) that leads to a newly built woven bone formation, which is frequently noted macroscopically as bone apposition (Maat, 1982; Schultz, 1993, 2001; Ortner, 2003). Only microscopic investigation reveals a reliable diagnosis (Schultz, 2001, 2003). Thus, in this child, the chronic course of scurvy resulted in a secondary immunodeficiency (Schmidt-Schultz and Schultz, 2004).

For the first time, we have now identified several typical growth factors in ancient human bone, such as BMP-2, TGF-β and IGF-II, using recent human bone and tooth dentin as a control. The identification of BMP-2 and IGF-II was also checked using their specific blocking peptide on the basis of several faint bands in Western blots.

Growth factors are fixed and stored in large amounts in the mineralized matrix of bone for future actions. According to this model, growth factors such as IGF-II are deposited for a time and then released by osteoblast bone resorption in a bioactive form to act on preosteoblasts and mature osteoblasts, thus allowing for site-specific replacement of bone that is lost to resorption (Dequeker et al., 1993; Mohan, 1993).

Our results prove that a special matrix protein identified in recent bone or tooth can also be detected in ancient human bone or tooth from its typical molecular mass and identified by the same specific antibodies. The bone matrix proteins extracted and solubilized from archaeological bone samples and presented in this study were protected over a long period of time by the physical durability of bone. Thus, no significant diagenesis or extensive decomposition took place. Since we analyzed archaeological bone samples that were first examined histologically to study the microstructure, no proteins were extracted and no bands were stained by silver in the SDS-PAGE if pronounced diagenesis and/or severe decomposition of the organic matrix had destroyed the normal microstructure.

Over the last 20 years, there have been several publications dealing with the topic of proteins in ancient bones and there are different approaches to the investigation of ECM proteins in ancient bone. As a rule, these research groups hydrolyzed ECM bone proteins and determined the amino acids by automatic analyzer with the aim of diagnosing the features of porotic hyperosto-

**Table 2** Matching of human ECM proteins from compact bone (early Middle Ages) and human ECM proteins from dentin of tooth 13 (early Middle Ages).

| Gel | Replicate groups | Spots | Matched | Match rate (%) | | Correlation coefficient |
|---|---|---|---|---|---|---|
| | | | | 1 | 2 | |
| Zahn-B GS 01 v1 | NA | 335 | 332 | 99 | 100 | 1.000 |
| Knochen-B GS 01 v1 | NA | 338 | 56 | 16 | 16 | 0.122 |

Match Set with PDQuest 2-D 7.0 from Bio-Rad. Zahn-B GS 01 v1, tooth dentin; Knochen-B GS 01 v1, compact bone; NA, not assigned.

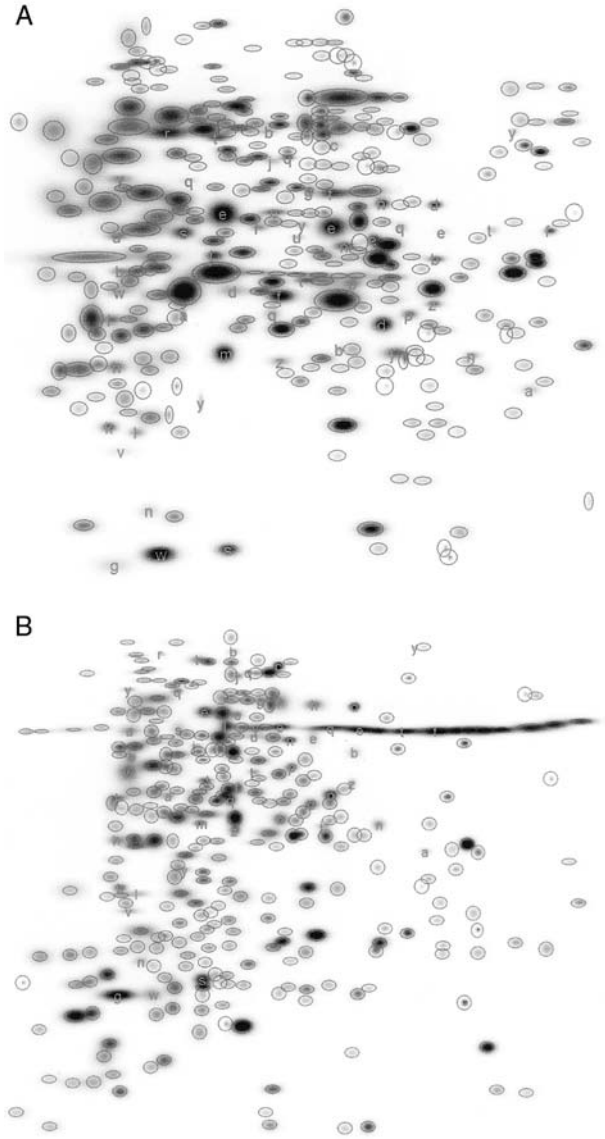


**Figure 7** Matching of human ECM proteins from compact bone and dentin.
(A) Matching of human ECM proteins from compact bone (early Middle Ages) separated by 2-D electrophoresis and stained with silver. The letters mark protein spots that are identical in compact bone and dentin of tooth 13 from the same individual; circles mark protein spots found only in compact bone (PDQuest 2-D 7.0, Bio-Rad).
(B) Matching of human ECM proteins from dentin of tooth 13 (early Middle Ages) after 2-D electrophoresis and staining with silver. The letters mark protein spots that are identical in dentin of tooth 13 and in compact bone from the same individual; circles mark protein spots that were only found in dentin of tooth 13 (PDQuest 2-D 7.0).

sis (Van Endt and Ortner, 1982; Grupe, 1995). However, this represents very superficial analysis with no possibility of obtaining a reliable diagnosis at the molecular level. Other research groups looked for so-called blood proteins (Catteano et al., 1990; Grupe and Turban-Just, 1996), for example albumin. Albumin is a protein synthesized by liver cells and transported by the blood through organs and tissues and to the bone, where albumin is a normal component of the bone matrix (Quelch et al., 1984). It is well known that the components of the blood are the first to be degraded by decomposition after death. Thus, as a rule, there is almost no way to detect blood proteins in archaeological bone.

Some research groups have tried to identify specific proteins with antibodies (Smith and Wilson, 1990; Brandt et al., 2002) in a crude smear extract of bone ECM. Such a smear of crude bone extract may contain many different structures, such as apatite, lipids and all the proteins attached to them, and which may be smaller or larger than 10 kDa. In a smear, these proteins which are, as a rule, peptides, are not solubilized. This is a crucial condition, because then the exact separation of these proteins in SDS-PAGE and their detection by specific antibodies is not possible. The precondition for the exact separation and identification of bone matrix proteins using specific antibodies in Western blot or ELISA is that all the proteins are really solubilized (Merril and Washart, 1998). This can be proved by a clearly separated protein pattern from SDS-PAGE stained with silver (as shown in Figure 2). This procedure is important to obtain reliable results using specific antibodies.

Another approach to the determination of ancient molecules was explored by M.J. Collins and his group, who looked for the degraded products of such molecules and created interesting mathematical models for their breakdown (Collins et al., 1995; Collins and Riley, 2000; Wess et al., 2001).

The human bone matrix consists of approximately 200 different proteins (Boskey, 1992). 2-D electrophoresis is the most valuable separation method for resolving complex protein mixtures (proteomics) based on charge, using isoelectric focusing (IEF) in the first dimension and SDS-PAGE to determine molecular weight in the second dimension. If we consider that the matrix protein pattern also contains several isoenzymes, we can solubilize most if not all human bone matrix proteins using the method described (Schmidt-Schultz and Schultz, 2004). There are no data in the literature on the number of matrix proteins in human tooth dentin. However, it is possible that the number is in the same range as in human bone matrix, i.e., approximately 200.

Macroscopically, the main protein spots and the protein pattern differ greatly between bone and tooth dentin and only 16% of protein spots in the tooth dentin were also found in bone from the same individual. This is the first result that deals with the matrix protein pattern of bone and tooth dentin from the same individual. In the near future, we will compare more individuals to gain a better overall view in this field.

Knowledge of all bone matrix components is a prerequisite for understanding the biochemistry and physiology of bone. This is also the basis for discussion on the state of bone disease. We now have the potential to identify the ECM proteins of ancient bone step by step. This offers a good opportunity to gain more insight into the new field of molecular paleopathology. Morphological investigations and biochemical results are complementary in making reliable diagnoses of diseases. Thus, for example: (i) the occurrence of characteristic macroscopic and microscopic bone alterations caused by tuberculosis (cf. Ortner, 2003; Schultz, 2003) and the detection of antibodies against tuberculosis in ECM from the same spec-

imen; or (ii) the occurrence of characteristic macroscopic and microscopic alterations due to tumorous diseases (Ortner, 2003; Schultz, 2003) and the identification of specific tumor markers, such as prostate specific antigen (Oremek et al., 2003), will provide very trustworthy diagnoses. Specific matrix protein groups, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-$\alpha$ are typical of acute inflammation, whereas interferon (IFN) in combination with TNF-$\alpha$ is more characteristic of chronic inflammation (Goldsby et al., 2000; Nanes, 2003). We have already detected some of these above-mentioned molecules in ancient and recent bone tissues (data not shown). Furthermore, we have a control-collection of recent pathologically changed bones at our disposal for which the diagnoses of diseases are known.

Within the near future it should be possible to increasingly understand the characteristics of particular diseases observable in bones and teeth from ancient times. This will go some way towards filling in the gaps in our knowledge of the evolution of human health and disease. Future efforts to understand past diseases will be enhanced greatly by this newly emerging technology.

## Materials and methods

### Preparation of thin ground sections for microscopic examination

After documentation (photographs, drawings and measurements), thin ground sections of bone samples were prepared by suitable techniques (Schultz and Drommer, 1983; Schultz, 1988, 2001).

### Protein extraction

ECM proteins from compact (cortical) bone and tooth dentin were extracted as previously described (Schmidt-Schultz and Schultz, 2004) using the method of Termine et al. (1980) with modifications. The external surfaces of the bone were carefully cleaned mechanically and by treatment with ultrasonication. From the dentin, the cement layer was removed. After drying, the bone or dentin sample was powdered under permanent cooling. Approximately 1 g of mineralized bone powder was extracted with 5 ml of buffer A (4 M guanidine-HCl, 20 mM $NaH_2PO_4$, 30 mM $Na_2HPO_4$, pH 7.4) under permanent stirring for approximately 1 day (4°C). After centrifugation (10 000 *g*, 30 min, 4°C), the supernatant was removed. The pellet was extracted with 5 ml of buffer B (buffer A and 300 mM EDTA) under constant stirring for 1 day (4°C). From the resulting extract, chelated calcium ions were removed by washing (35 000 *g*, 20 min) three times with autoclaved double-distilled water. The pellet, containing the bone matrix extract (BME), was lyophilized. BME can be stored for several months at -20°C. Each extraction step was carried out with a combination of protease inhibitors, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 mM aprotenin, 50 $\mu$M leupeptin and 10 mM EDTA. All procedures were performed using gloves, autoclaved instruments and autoclaved or sterile filtered solutions.

### Solubilization, precipitation and separation of proteins

Approximately 20 mg of BME was sonicated twice in ice for 7 s in a neutral 30 mM phosphate buffer with the protease inhibitors 2 mM aprotenin, 1 mM benzamidine, 10 mM $\varepsilon$-aminocaproic acid and 10 mM EDTA. After solubilization, proteins were precipitated with trichloroacetic acid (8% final concentration) and separated by SDS-PAGE according to Laemmli (1970).

### Western blot analysis

The proteins were identified by Western blotting with specific antibodies. Denatured protein samples were separated by SDS-PAGE with 14% total acrylamide (T) and 2.5% cross-linker (C), then transferred to polyvinylidene fluoride (PVDF) membranes in a Trans-Blot cell (Bio-Rad) with water-cooling for temperature control. The non-specific binding sites were blocked with 5% non-fat dried milk and incubated with one of the following primary antibodies: anti-human TGF-$\beta$ (diluted 1:600; Chemicon, Harrow, UK), anti-human BMP-2 (diluted 1:200; Santa Cruz, Santa Cruz, CA, USA), or anti-human IGF-II (diluted 1:150; Santa Cruz). Horseradish peroxidase-linked anti-rabbit (1:50 000) or anti-goat (1:2000) (Santa Cruz) was used as the secondary antibody. Bands were visualized using an enhanced chemiluminescence (ECL)-Plus detection system.

The controls for BMP-2 and TGF-II were run with their blocking peptides (Santa Cruz).

### Two-dimensional electrophoresis

The solubilized proteins were loaded onto a dehydrated and immobilized pH gradient (IPG) 7-cm strip, pH 3–10. Focusing was performed at a maximum of 20°C (Protean IEF-cell, Bio-Rad), 4000 V and 10 000 V h. After the focusing procedure, the strips are incubated with 2% dithiothreitol in equilibration buffer (6 M urea, 0.375 M Tris, 2% SDS, 20% glycerol) for 10 min to reduce the proteins. These are then carbamidomethylated for a further 10 min (260 mM iodoacetamide in equilibration buffer). Equilibrated IPGs were transferred to a polyacrylamide gel (12% T, 2.5% C) without stacking gel. The SDS gel was run according to Laemmli (1970). Two-dimensional-gels were stained with silver according to the protocol of Swain and Ross (1995).

### PDQuest 2-D 7.0 analysis software

PDQuest 2-D 7.0 software (Bio-Rad) was used to count the silver-stained spots in 2-D electrophoresis and to match the 2-D gels of bone and dentin from the individual dating from the early Middle Ages (Barbing, 450–600 AD).

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