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CALCITONIN PRODUCING TUMOUR

Effects on Fracture Repair and Normal Bone in Rats

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The mechanical properties and the collagen metabolism of healing fractures and intact bones have been studied in rats with a transplanted, calcitonin (CT) secreting, medullary thyroid carcinoma (MCT). Sham operated animals served as controls. The MCT was transplanted beneath the kidney capsule. Seven months later, when the rats with MCT had increased circulating levels of CT, a standardized femoral fracture was produced in all the animals.

The serum levels of CT were 3–40 times higher in tumour bearing rats than in controls in the period following the fracture. The fracture strength of rats with MCT was reduced by about 60 per cent compared to controls at 16 weeks after the fracture. The strength of intact femora (ultimate torsional moment) seemed to be progressively impaired by increasing levels of circulating CT. Also the strength of bone as a material (ultimate torsional stress) was reduced in the rats with MCT.

The collagen synthesis was reduced in MCT rats, but the amounts of collagen in fractured or intact bones were not changed compared to controls.

We conclude that chronic hypercalcitoninaemia due to MCT seems to have a negative influence both on fracture healing and on bone metabolism.

Key words: biomechanics; bone development; ^{14}C-hydroxyproline; callus; collagen; femur; growth; medullary thyroid carcinoma; stress

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The thyroidal hormone calcitonin (CT) is claimed to stimulate bone formation (Duriez 1979), but there is conflicting information regarding its therapeutic value in the treatment of fractures (Bastiani et al. 1974, Messina et al. 1974) and of postmenopausal osteoporosis (Jowsey et al. 1971, Chesnut et al. 1979, Zanzi et al. 1981).

We have previously reported that salmon CT has a negative influence on the collagen metabolism of healing fractures in young rats (Ekeland & Underdal 1982). The CT employed was, however, from another species, and the circulating levels of the administered drug changed considerably during the day, as evaluated by its effect on serum calcium. Inbred Wag/Rij rats with a transplanted, CT secreting, medullary thyroid carcinoma (MCT) have increased levels of circulating, endogenous CT (Boorman et al. 1974, Normann et al. 1977, Ekeland et al. 1980, 1981b). In a previous study, the transplanted MCT neither metastasized nor affected the general condition of the animals (Ekeland et al. 1980). The tumour derived CT secretion was also sensitive to normal secretagogues (Normann et al. 1977). Moreover, CT from MCT was found to be biochemically similar to normal thyroidal CT and biologically active (Myhre et al. 1979, Ekeland et al. 1980). Thus, Wag/Rij rats with a transplanted MCT should represent a suitable model to investigate the effects of increased, circulating, endogenous CT.

The purpose of the present experiment was to

study fracture repair and bone metabolism in these MCT rats with chronic hypercalcitoninaemia, using both mechanical and chemical methods.

MATERIALS AND METHODS

Experimental animals

Forty-eight inbred, Wistar derived, male Wag/Rij rats, 24–31 days old, and weighing about 50 g, were divided into two weight matched groups of 24 animals. In one group, a specimen from a rat MCT was transplanted underneath the capsule of the left kidney, using ether as anaesthesia (Boorman et al. 1974, Ekeland et al. 1981b). The transplanted tumour had previously been cultured *in vivo* in successive generations of rats, and the tumour specimens used in the present experiment represented the fifth generation. The control rats were sham operated. Three rats were housed in each cage, and they were given standard animal pellets containing 0.9 per cent calcium and 0.7 per cent phosphorous (Bl. nr. 3155, Møllesentralen i/s, Oslo, Norway), and water *ad libitum*.

The growth of the transplanted tumour was monitored by measuring the circulating levels of CT. Blood samples were collected from the rats' tails (Ekeland et al. 1981b). Six months after the transplantation, the plasma CT had increased to three times the levels in the control rats (Figure 1). One month later, just before the experimental fracture was induced, the rats with MCT and the controls were pair matched according to body weight. They were divided into four groups, A, B, C and D, on the basis of increasing weight. Each group consisted of six transplanted and six control rats.

Experimental fracture and mechanical testing

Seven months after the transplantation, a standardized, left, mid-femoral fracture was produced in all the rats during ether anaesthesia, as previously described (Ekeland et al. 1981a). The rats walked on the unimmobilized, fractured limb within a few days.

At 3, 6, 12 and 16 weeks after the fracture, rats from groups A, B, C and D were weighed, anaesthetized with

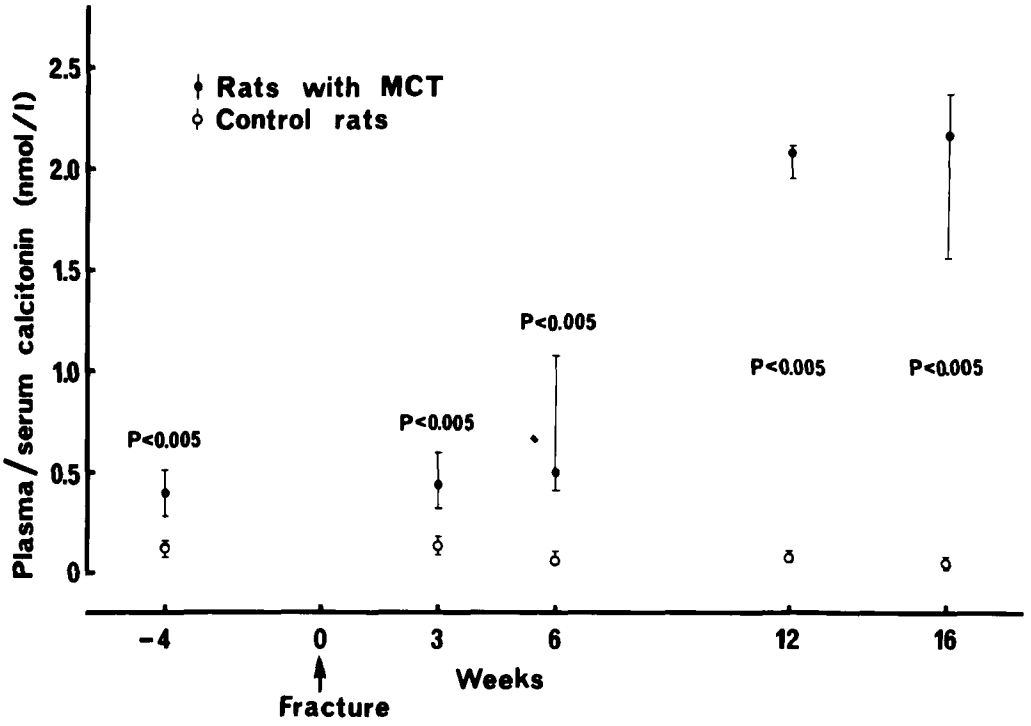

Figure 1. Circulating levels of calcitonin (CT) in rats with a transplanted, CT secreting, medullary thyroid carcinoma (MCT) and in controls. The rats were given a left femoral fracture 7 months after the MCT transplantation. CT was analysed in plasma 4 weeks before the fracture (24 animals in each group), whereas the postfracture CT analyses were performed in serum (six animals in each group). The CT levels are higher in rats with MCT than in controls, and the levels of significance are indicated. (Median with 0.25- and 0.75-fractiles).

ether, and killed by aortic exsanguination. The collected blood was centrifuged, and the serum stored at −20°C until analysed for CT, calcium, phosphorous and albumin. The transplanted MCT was removed along with the left kidney. The abdomen and the thorax showed no signs of metastases. Both femora and the right tibia were immediately dissected free and stored in cooled isotonic Ringer's solution. The length of intact femora was measured with a sliding caliper and within 4 h, both fractured and intact femora were tested in torsion until failure (Engesæter et al. 1978, Ekeland et al. 1981a).

The *ultimate torsional moment,* defined as the strength of the bones (Ekeland et al. 1981a), was obtained from the load-deformation curves along with the *torsional stiffness* and the *ultimate torsional angle* (Ekeland et al. 1982). The *fracture strength* was defined as the torsional moment necessary to twist the fractured bones 20 degrees (0.35 rad), since this value gives more accurate information on the degree of fracture repair than the ultimate torsional moment of the healing fractures (Ekeland et al. 1982). The strength and the stiffness of bone as a material, the *ultimate torsional stress* and the *modulus of rigidity,* were calculated for intact femora in group D after the major and the minor diameters and the cortical thickness of the bones had been measured (Ekeland et al. 1982).

The tested femoral fractures and the tibiae of rats from Groups A and B were stored at −20°C until analysed for collagen.

Collagen analyses

Twenty-four hours before the rats in group A and B were killed, 15 μCi (0.56 M Bq) L(U-^{14}C) proline (295 mCi (10.9 G Bq)/mmol) (The Radiochemical Centre, Amersham, Buckinghamshire, England) per 100 g body weight was injected intraperitoneally. The content and the synthesis of collagen in fractured and intact bones were assessed by measuring the amounts of hydroxyproline, respectively ^{14}C-hydroxyproline in specimens from these bones (Firschein 1969). The specimens from healing fractures were prepared from the middle portion of the fractured femora. The proximal 7.5 mm of the bone was removed using a saw, and the distal epiphysis using a forceps. Thereafter, the soft tissues were peeled off the specimens without removing any parts of the callus. The specimens of intact bones were made from the proximal metaphysis of right tibiae by removing the knee-near epiphysis along with the soft tissues. The proximal 10 mm of the remaining bone was then separated from the distal part using a saw, and used for further analyses. These have been described in detail previously (Firschein 1969, Ekeland & Underdal 1983), and include determination of the dry weight, the total content, the concentration and the synthesis of collagen in the bone specimens.

Analyses in blood

CT was assayed radioimmunologically in plasma or serum using rabbit antisera directed against both N-terminal and C-terminal amino acid sequences of the human hormone (Gautvik et al. 1976, Myhre & Gautvik 1979). As rat CT and human CT have a similar structure (Raulais et al. 1976), this assay has proved to be a reliable measure also for rat CT (Normann et al. 1977, Ekeland et al. 1980).

Serum levels of calcium, phosphorous and albumin were measured by means of an Abbott Bichromatic Analyzer, as previously reported (Ekeland & Underdal 1983).

Statistical analyses

Median with 0.25- and 0.75-fractiles were used to express the average and the dispersion of the measured values. The statistical significance probability was calculated by Wilcoxon's two-tailed test for two samples (Diem & Lentner 1975). However, to evaluate differences in circulating CT between rats with MCT and controls, Wilcoxon's one-tailed test for two samples was used. Differences were considered significant when $2P \leqq 0.05$ (two-tailed test) and $P \leqq 0.05$ (one-tailed test).

RESULTS

Growth of the animals

The body weight of the rats increased steadily until their left femur was fractured, without any significant differences between MCT transplanted and control rats. At the time of the fracture, the body weight of rats with MCT was 331 g (fractiles 322–342 g), and that of controls 338 g (fractiles 326–348 g). Following the fracture, however, the rats with MCT lost 1–7 per cent more weight than the controls. After 16 weeks, the control rats had regained, and the rats with MCT had almost regained the prefracture weight.

The differences in femoral length between rats with MCT and controls were small and mostly insignificant. The dry weight of standardized specimens from fractured femora was lower in rats with MCT compared to controls 3 weeks after the fracture (Table 1). No differences were, however, observed in the dry weight of standardized specimens of intact bones (Table 2).

*Table 1. Dry weight of standardized specimens from healing femoral fractures in rats with a transplanted, calcitonin (CT) secreting, medullary thyroid carcinoma (MCT) and in controls. Also the total content, the concentration and the synthesis of hydroxyproline (Hyp) in the specimens are given. Significant differences between rats with MCT and controls are indicated by asterisks. **2P = 0.02, *2P = 0.05. Six animals in each group. (Median with 0.25- and 0.75-fractiles)*

Weeks after fracture	Group tested	Dry weight (mg)		Total content of Hyp (μmol)		Concentration of Hyp (nmol/mg bone)		Specific activity of ^{14}C-Hyp (DPM/nmol Hyp)	
		Rats with MCT	Controls	Rats with MCT	Controls	Rats with MCT	Controls	Rats with MCT	Controls
3	A	529 (513–533)	* 566 (548–567)	81.2 (78.0–83.4)	84.6 (83.1–87.3)	153 (149–156)	151 (149–153)	0.86 (0.82–0.91)	** 1.03 (0.96–1.11)
6	B	586 (525–609)	611 (593–633)	96.5 (83.1–100.8)	100.7 (94.6–102.6)	163 (159–168)	167 (158–173)	0.50 (0.42–0.55)	0.55 (0.51–0.57)

*Table 2. Dry weight of standardized specimens of intact bone from proximal tibia metaphyses of rats with a transplanted, calcitonin (CT) secreting, medullary thyroid carcinoma (MCT) and of controls, following fracture of the contralateral femur. Also the total content, the concentration and the synthesis of hydroxyproline (Hyp) in the specimens are given. Significant differences between rats with MCT and controls are indicated by asterisks. *2P < 0.05. Six animals in each group. (Median with 0.25- and 0.75-fractiles)*

Weeks after fracture	Group tested	Dry weight (mg)		Total content of Hyp (μmol)		Concentration of Hyp (nmol/mg bone)		Specific activity of ^{14}C-Hyp (DPM/nmol Hyp)	
		Rats with MCT	Controls	Rats with MCT	Controls	Rats with MCT	Controls	Rats with MCT	Controls
3	A	132 (124–139)	128 (124–131)	16.6 (15.9–18.3)	16.1 (15.6–17.2)	130 (128–132)	128 (124–133)	0.33 (0.30–0.36)	0.33 (0.30–0.34)
6	B	140 (139–142)	139 (135–140)	17.1 (16.3–17.5)	17.2 (16.8–17.9)	126 (123–130)	126 (124–129)	0.22 (0.19–0.23)	* 0.29 (0.25–0.31)

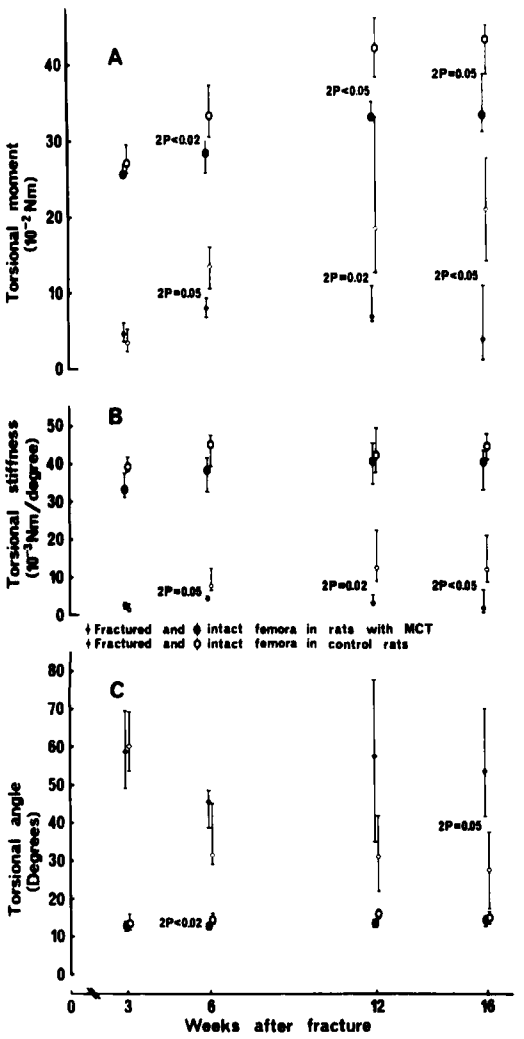

Figure 2. Strength (A), torsional stiffness (B) and ultimate torsional angle (C) of fractured and intact femora from rats with a transplanted, calcitonin (CT) secreting, medullary thyroid carcinoma (MCT) and from controls. For intact femora, the ultimate torsional moment is plotted as strength. For fractured femora, the torsional moment required to twist the fractures 20 degrees (0.35 rad) is plotted as strength. Six animals in each group. Significant differences between rats with MCT and controls are indicated by P-values. (Median with 0.25- and 0.75-fractiles).

Analyses in blood

Circulating levels of CT increased with time in rats with transplanted MCT, reaching concentrations about 40 times those of control rats at the end of the study (Figure 1). In MCT rats the CT levels seemed to be related to the size of the transplanted tumour, reflecting the tumour growth.

There were no significant differences in the serum levels of calcium, phosphorous and albumin between rats with MCT and controls, but serum calcium in both groups increased from about 2.45 mmol/l to 2.70 mmol/l during the study.

Mechanical bone tests

Fractured femora. From the 6th week following the fracture, the strength and the stiffness of the fractures in rats with MCT were lower, and the deformability (ultimate torsional angle) was larger than corresponding values in control rats (Figure 2). At 16 weeks, these three biomechanical parameters were of the same magnitude in rats with MCT as observed 3 weeks following the fracture, possibly as a sign of pseudarthrosis development. None of the fractures were at any time consolidated as evaluated manually, and the fracture strength at the end of the experiment was reduced by about 60 per cent compared to controls. The fracture healing in control rats seemed to progress slowly with time, and only half of the fractures were consolidated 12 and 16 weeks after the fracture.

Intact femora. The strength of femora from rats with MCT was progressively reduced compared to controls as the serum levels of CT increased (Figures 1 and 2). Similar observations were made regarding the torsional stiffness and the ultimate torsional angle of the bones, but here the differences between the two groups of rats were less pronounced (Figure 2). At 16 weeks after the fracture, the strength of bone as a material, the ultimate torsional stress, was significantly reduced ($2P<0.05$) in femora of tumour bearing rats ($2.57 \cdot 10^7$ N/m^2) compared to those of controls ($3.15 \cdot 10^7$ N/m^2). This reduction in bone material strength was about 20 per cent, and comparable to the strength reduction of intact femora at that time. No significant difference was, however, observed in the modulus of rigidity

of the bone material from the two groups of rats (values about $18 \cdot 10^8$ N/m^2 rad at 16 weeks after the fracture).

Collagen analyses

The collagen synthesis in fractured bones was significantly reduced in rats with MCT compared to controls 3 weeks after the fracture (Table 1). Corresponding observations were made in intact bones 3 weeks later (Table 2). In contrast, the total content and the concentration of collagen in the bone specimens seemed to be uninfluenced by the transplanted tumour (Tables 1 and 2).

DISCUSSION

The present study revealed a negative influence of the CT secreting MCT on bone strength and collagen metabolism in rats.

The strength of rat femora is closely correlated to the body weight of the animals (Saville & Smith 1966, Ekeland et al. 1981a, 1982). In mechanical tests it is therefore important to use rats with small dispersion in body weight. Accordingly, the rats with MCT and the controls were pair matched into four test groups, each with slightly different median body weight.

The weight loss following the fracture was increased in rats with MCT. In a previous study with corresponding rats without fracture (Ekeland et al. 1980), the body weight of the rats was not significantly influenced by the tumour during an experimental period of 10 months. MCT rats may therefore have more local fracture symptoms, or they may be less resistant to stress situations generally.

CT is a hypocalcaemic hormone, but there were no detectable differences in serum calcium between the rats with the CT secreting MCT and the controls. This may be due to a secondary hyperplasia of the parathyroids (Normann 1977, DeLellis et al. 1979), since circulating CT from MCT rats induced hypocalcaemia when tested in a bioassay (Myhre et al. 1979, 1982). Serum ionized calcium, which is the physiologically important fraction of serum calcium (Bringhurst & Potts 1979) and more sensitive to CT injections (Myhre 1980), was, however, reduced in rats with transplanted MCT compared to controls in a previous study (Ekeland et al. 1980). As there were no significant differences between the serum albumin of rats with MCT and controls in the present study, the general condition of the tumour transplanted rats was probably not seriously affected during the experiment.

The negative influence of the CT secreting MCT on fracture repair is in accordance with the previously reported effects of salmon CT on the healing of femoral fractures in rats (Ekeland & Underdal 1983). The fractures in half of the control rats in the present experiment were not consolidated after 16 weeks. This is probably related to the age of the animals, since in a previous study, corresponding fractures in younger adult rats healed in 12 weeks (Ekeland et al. 1982). Also the mechanical properties of intact bones were impaired by the MCT (Figure 2). This cannot solely be due to the slightly lower body weight of the rats with MCT compared to the controls, since the strength of bone as a material (e.g. the ultimate torsional stress) was also significantly reduced in rats with the CT-producing tumour. The ultimate torsional stress of intact femora has previously been shown to be independent of age and body weight in adult rats (Ekeland et al. 1982).

The incorporation of radioactive proline into bone and its conversion to hydroxyproline is an accepted method to measure the collagen synthesis in bone tissue (Laitinen 1967, Firschein 1969). The activity of ^{14}C-hydroxyproline in the bone specimens in our study indicates the net synthesis of collagen during a 24-h period. The collagen analysis of intact bone was made in specimens from the proximal tibial metaphysis, since in a preliminary study, the collagen synthesis was found to be about 80 per cent higher in this region than in the distal femoral metaphysis. Similar findings have been reported by Firschein (1969). As observed after the administration of salmon CT to Wistar rats (Ekeland & Underdal 1983), the collagen synthesis in fractured bones of MCT rats was transiently reduced compared to controls 3 weeks after the fracture (Table 1). Similar observations were made for intact bones 3 weeks later (Table 2). Since the total content

and concentration of collagen in the bone specimens did not decrease, bone resorption was probably concomitantly reduced in the rats with the CT-producing tumour. Other studies have shown a decreased urinary excretion of hydroxyproline (Ekeland et al., in preparation) and a reduced calcium concentration of intact tibiae in these rats (Myhre et al. 1980). Accordingly, a reduced bone remodelling where the mineral accretion is impaired, may result in hypomineralization, and possibly explain the reduced strength of the bones in the tumour-bearing rats. Both a reduced (Melvin et al. 1973), and a slightly increased (Emmertsen et al. 1982) bone remodelling has been reported in humans with MCT, but the biological activity of circulating CT in such patients is questionable (Myhre et al. 1979, Myhre & Gautvik 1979).

MCT has also been reported to secrete other peptides than CT (Shibasaki et al. 1979). Our observations may therefore have been influenced by effects of the transplanted tumour separate from those caused by CT. It is, however, reasonable to believe that the increased production of endogenous CT played an important role, since the bone metabolism was impaired in a similar way after administration of salmon CT to rats (Ekeland & Underdal 1983), whereas bone mineralization was unaffected in rats with other peptide secreting tumours (e.g. prolactin) (Myhre et al. 1981).

In conclusion, a transplanted, CT secreting MCT in rats had a negative influence both on fracture repair and on metabolism of intact bones. At the end of the experiment, the fracture strength and the strength of intact bones in MCT rats were reduced by about 60 and 20 per cent, respectively, when compared to controls. The MCT effects on intact bones seemed to increase with increasing levels of serum CT.

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