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RADIAL RATE OF OSTEON CLOSURE, MEASURED BY MEANS OF TETRACYCLINE LABELLING

O. Landeros, M.D., and H. M. Frost, M.D.

Introduction

There is little information in the literature of diabetes mellitus concerning the physiology of lamellar bone. In 17 patients with diabetes mellitus, Klein and Frost suggested two things: that there is a major abnormality in the remodeling of bone in this disease, which deserves further study, and that such study offered hope of gaining insight into diabetic cell dynamics, a subject of which little or nothing is known.[1,2] Among the problems posed by extending their study is that of characterizing whatever kinds of cell behavioral disturbances may exist in making new bone in the skeleton. It has long been known that lamellar bone formation in the human adult occurs in physically and temporally circumscribed units or foci.[3] This means that the total amount of new bone that is formed in a given period of time is a function of (among other things) the speed with which the average, single focus of new bone formation is completed.[3]

This speed of completion is evaluated in this study by measuring the average rate at which layers of new organic matrix are added to previously formed matrix at individual foci of osteon (i.e., Haversian system) formation. This is called the radial rate of osteon closure. The measurements were done on ribs taken from 11 patients with diabetes mellitus, and involved measuring the widths of tetracycline labels deposited in these ribs *in vivo*.[4,5]

Osteon formation is a centripetally progressive process, so it may be termed osteon closure. The study revealed a major alteration in the parameter that was measured in diabetics.

Materials

Eleven patients with diabetes mellitus were available who had been labelled one or more times with a tetracycline antibiotic in a manner which made them suitable subjects for the measurements. Their mean age was 65 years and they supplied 13 bones for study. The cases are listed in Table I, along with the measurements obtained from each, and with other information pertinent to the study. The number of months between labelling and skeletal sampling is indicated in column four of this table and averages 27 months. Column five shows whether or not the patient was in reasonably good health at the time of labelling.

Table I

Case	Sex Age	Bone and Sections	Months between label and sampling	Ill at time labelling	No. labels measured	Microns/day Mean MF.	Microns, range
958882	52 F	2 cls. 2 ribs	At death	Yes	13	.261	0-.757
343203	52 M	2 cls. 2 ribs	19 mos.	No	21	.212	0-.617
316566	57 M	7 cls.	9 days	Yes	76	.179	0-0.411
948209	58 F	6 ribs	8 days	Yes	32	.057	0-.256
1032733	59 F	3 ribs	3 days	Yes	15	.223	.437-1.29
905186	59 M	3 ribs	6.5 mos.	No	13	.270	0-1.12
293647	60 M	4 ribs	18 mos. 17 mos.	 No	14	.283 .063	.143-.483 .042-.083
211077	73 F	2 cls.	9 years	No	11	.290	0-.636
810770	80 F	3 ribs	6 mos.	Yes	6	.143	0-.327
574244	81 M	3 ribs	6.7 yrs. 3.0 yrs.	No No	17	.387 .291	0-.924 .054-.641
779588	82 M	6 ribs	8 mos.	No	16	.086	0-.169
Means	64.8		22		234	Mean MF = .210	
(Cl: clavicle)	64.8	45	27.1		234	SD .099 SE .028	0-1.29

The cases that were studied are listed, with data pertinent to the study and the text. The wide range in single values in individual cases that appears in the right hand column is characteristic of this measurement. It may mean that there are large fluctuations in the local rate of bone formation in time periods on the order of a week or two. It is significant that the means for individual cases do not show a variation of this magnitude with respect to the group mean.

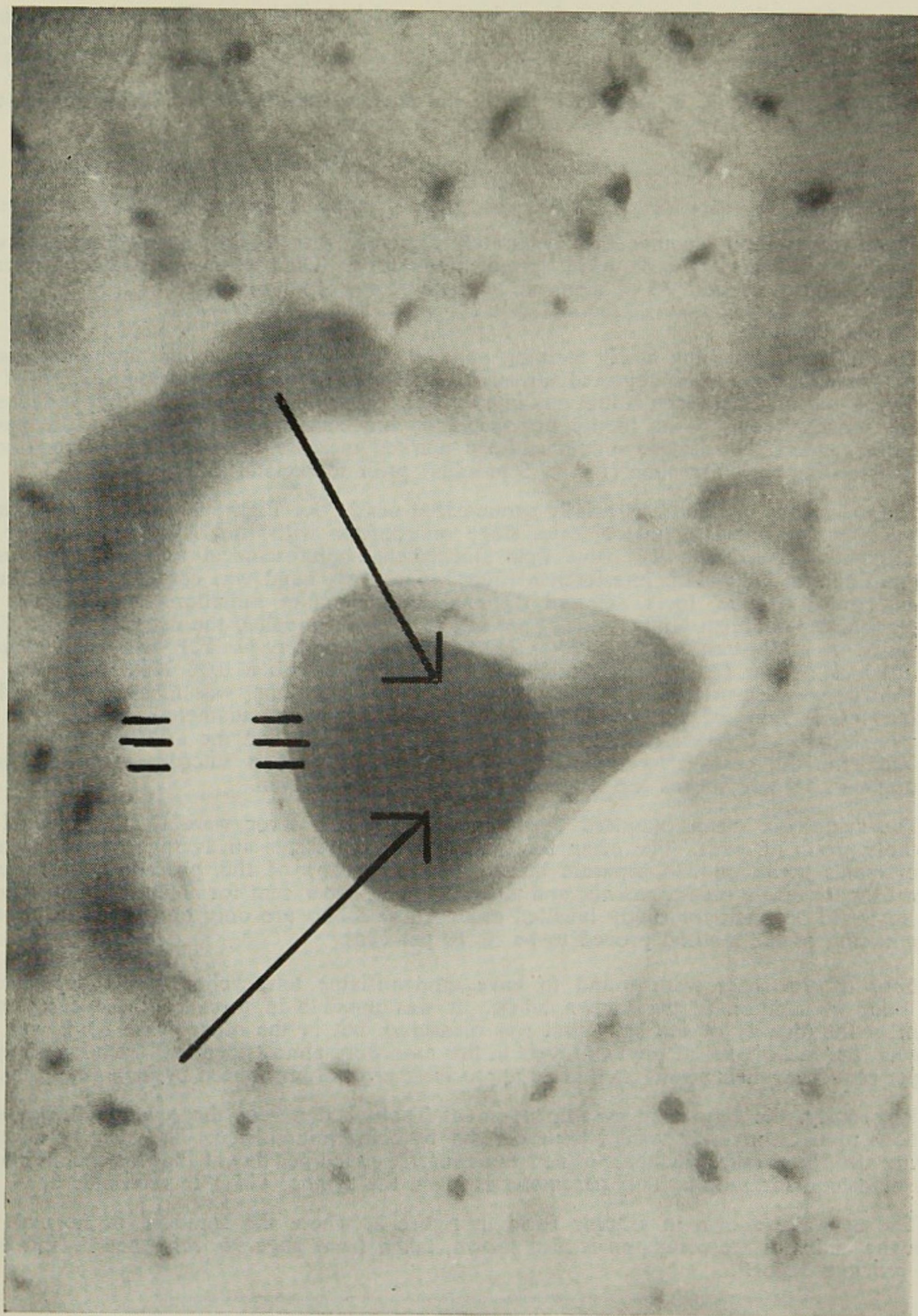

Figure 1

The India ink marks bracket a double tetracycline label deposited in an actively forming osteon some months before this bone was obtained. Mineralized, hand ground section, blue-light flourescence, 300 X. The arrows show the direction in which new bone formation proceeded while the label was being deposited. The thickness of the label is due to two additive things: the number of days during which the tetracycline antibiotic was given, and the thickness of the label being actively deposited at any instant. (TISZ).

In all cases, the antibiotics were given for infections or febrile illnesses. The patients providing the normal standard with which the diabetic patients are compared were similarly labelled and for similar reasons.[5]

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Methods

Sections

Fresh, mineralized, unembedded, accurately oriented, basic fuchsin stained sections were made from the rib of each case, using special methods[6,7,8]. The sections averaged 50 microns in thickness and there were 45 of them, an average of over four per case.

Tetracycline Labelling

The details of labelling newly forming bone *in vivo* by a tetracycline antibiotic, and of its measurement, have been reported already[5]. An unremarked but valuable feature of the tetracycline marking technique is that any intelligence concerning cell behavior that is deposited with the marker seems to be totally unaffected by any subsequent disease that the patient may suffer, unless this disease should cause a marked increase in resorption or remodeling rates over a long period of time (i.e., $>$ 3 months) prior to skeletal sampling.

The measuring procedure is briefly summarized next[5]. See Figure 1. A Zeiss micrometer eyepiece was calibrated against a Zeiss stage micrometer with the objective used for the measurements. (Needham, 9). Blue light flourescence microscopical technique was used[5]. A 40 millimicron bandwidth interference filter, whose pass band was centered on 460 millimicrons (Baird Atomic, Inc.), selected the energizing band of radiation. A 100 watt, low voltage, tungsten filament lamp (Zeiss) operated at 12 volts supplied the necessary light. Two thicknesses of a Wratten 8 gelatin filter (E. Kodak, Rochester, N. Y.) over each eyepiece were used as barrier filters. Individual labels were found with a 0.32 N.A. objective, and their thicknesses measured with a 0.65 N.A. objective. Each label was measured at each of the four intersections of the labelled ring with two orthogonal diameters centered on the Haversian canal. All labels of known identity were measured, and the arithmetic mean then calculated for the case, after subtracting the TISZ value of 4 microns from individual measurements. These means are listed in Table I, column seven.

The arithmetic mean, standard deviation and standard error were then calculated for the whole group of cases. The mean for the 11 cases represents an average of 936 separate measurements made on 234 separate labels. The accurarcy of this procedure averages $\pm$ 20 per cent for single measurements, and varies from $\pm$ 5 per cent for some multiply labelled cases to $\pm$ 30 per cent for singly labelled cases whose labels are only of five days duration. The precision of the method proved to be $\pm$ 10 per cent.

Four of the cases were found to have unidentifiable tetracycline labels deposited in their bone in addition to the known labels. It was possible in three of these cases to be certain of the identity of the label that was measured, but in the other (case 6) it was not. However, the thicknesses of the two labels in this case were almost identical (relative variation $\pm$ 8 per cent from their mean) so that no appreciable error is introduced by including this case.

Rigorously, the figures in column seven of Table I represent the average thickness of new bone matrix converted to new bone per day by being mineralized. This may be accepted as being also the average thickness of new bone matrix added per day at the average, individual focus of osteon formation. This parameter is given the symbol (M_f) in reference 3.

The data are shown in graphic form in figure 2, where the standard of normal with which the diabetics are compared is also shown, taken from page 96, reference 3, and based on a previous report[5].

Results

The mean for the radial rate of osteon closure in 10 diabetic subjects is 0.077 $\pm$ 0.036mm/year (or 0.21 $\pm$ 0.099 microns/day). The scatter plot in Figure 2 shows that individual diabetic case values lay close to the mean.

The individual case means in Table I do not show any systematic variation in values between cases who were seriously ill at the time of labelling and those who

were not. No systematic difference occurs in the values of cases labelled years before skeletal sampling and those labelled within weeks of it. No systematic difference appears in the values according to age.

The large range in the values of single measurements that was found in single cases, and which is noted in column eight of Table I, seems to be characteristic of this kind of measurement.[5]

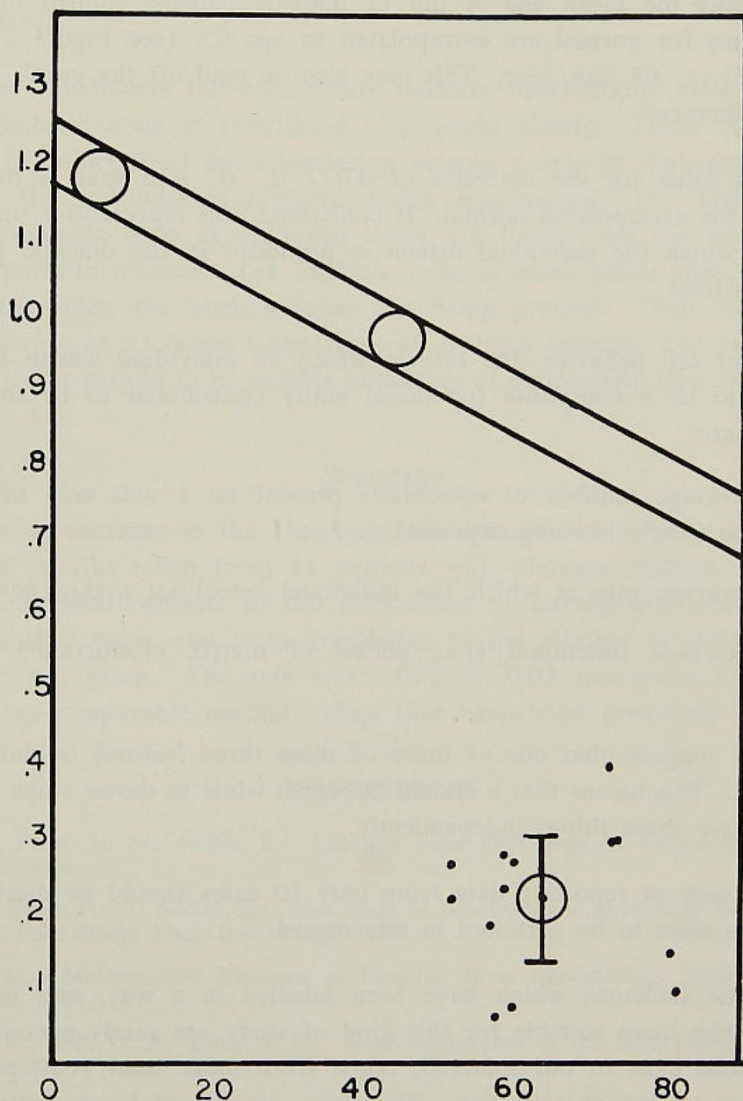

Figure 2

This graph shows the normal values for the radial rate of osteon closure at the top as a double line, widely spaced. The circles represent the two ages at which mean values were found.[5] The remainder of the slopes represent extrapolation. The scatter plot in the lower right of the graph shows the means of the individual cases in the diabetic study group. They cluster closely around the mean for all 11 cases. The bar shows one standard duration, the circle the standard error of the mean. The Y axis is in microns/day. The X axis is age in years.

Discussion

The radial rate obtained in this study is analogous to the rate at which depths of new ice are added to a skating rink by repeated flooding with water and then freezing.

A normal value for this rate in nondiabetics has been reported elsewhere to be approximately 0.9 ± .4 microns per day, or .33 ± .15mm/year[5], at age 47 years. An age-related decline was observed in normal people from age 7.5 to age 45, so it is likely that there is a further decline between age 45 and 65 in normals, the latter figure being the mean age of the 11 diabetic patients studied in this report. When the figures for normal are extrapolated to age 65, (see Figure 2) a value is obtained of 0.21 ± .08 mm/year. This may also be read off the graph in Figure 26 of the cited reference.[3]

The mean value for the diabetics of 0.077 ± .02 mm/year is therefore only 37 per cent of the extrapolated normal. If confirmed, this represents a major decrease in the rate at which the individual osteon is produced in the diabetic patient when compared to normal.

In terms of cell behavior, the rate at which an individual osteon is made may be considered to be a composite functional entity contributed to by three different things. These are:

1) The average number of osteoblasts present on a unit area of the surface where new bone matrix is being deposited.

2) The average rate at which the individual osteoblast makes new matrix.

3) The average functional (i.e., period of matrix production[3]) lifetime per osteoblast.

This study suggests that one or more of these three features is abnormal in the diabetic subject. This means that it should be worth while to devise ways of evaluating each one of these three things independently.

The legitimacy of reporting data from only 10 cases should be discussed briefly. Four comments seem to be pertinent in this regard.

1) Diabetic skeletons which have been labelled in a way, and under circumstances, that make them suitable for this kind of study are rarely encountered. Only 11 such skeletons exist in our material, taken from more than 1000 people over a 10-year span of work and collection. Therefore, reports of large numbers of cases in this kind of study cannot be expected.

2) The difference between the normal and the diabetic means are highly significant statistically. This is reflected in the t test, which shows that the probability of the null hypothesis being true is less than .001, i.e., $P < .001$ (with 24 degrees of freedom, and an estimated standard error of the difference between the normal

and diabetic means of .0866μ). This is true regardless of whether the normal standard is the 0.33 mm/year figure in reference 5, or the harsher, extrapolated value of 0.21 mm/year in reference 3.

3) Although all of the diabetic cases had serious illnesses in addition to their diabetes at the time of skeletal sampling, their skeletons were labelled an average of 27 months *before* skeletal sampling so their terminal illnesses did not tend to affect the measurements. In six instances, the patients were not considered seriously ill at the time of labelling, and so would be ideally suitable for comparison with the normal standard.

4) Other, qualitative but consistently uniform observations have long suggested to us that diabetic bone is remodeled abnormally slowly. These observations are three: a) The mineralized bone bordering osteoid seams in diabetics is much less permeable to the molecules of a standard dye than normal. (b) Tetracycline labels deposited in diabetic bone at unknown times are thinner on the average than are their counterparts in normals. (c) Diabetic bone is more brittle than normal, a fact routinely noted when the bone sections are being ground. These observations are based on a library of 93 bones taken from 40 diabetic subjects, and in other circumstances have so far proven to be reliable indicators of abnormally slow bone remodeling rates.

Summary

The rate of decrease in the Haversian canal radius of actively forming osteons was measured in ribs taken from 11 patients with diabetes mellitus. The rate was calculated from measurements of the thicknesses of tetracycline labelled bands deposited in lamellar bone, and from knowledge of the number of days during which the antibiotic was given. The rate was 0.077 ± 0.02 mm/year, a decline to 36 per cent of age-comparable normal values that have been previously established.

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