Quantitative acoustical assessment of wound maturation with acoustic microscopy

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Preliminary results of the ultrasonic characterization of cutaneous wound tissue and surrounding margin, obtained with the scanning laser acoustic microscope, show an increase in the speed of sound and in the acoustic heterogeneity as a function of wound age. As the wound age increased, the following results were noted: (1) The wound area, initially quite homogeneous in acoustic appearance, became more heterogeneous; (2) the acoustic appearance of the wound tended to become similar to that of the adjacent tissue; and (3) the ultrasonic velocity of the wound area increased from a range of 1540–1575 m/s at 7 days to a range of 1700–2000 m/s at 35 days.

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INTRODUCTION

Studies have suggested that collagen is an important tissue constituent in affecting ultrasonic propagation properties of tissue (Fields and Dunn, 1973; O'Brien, 1977; Goss and O'Brien, 1979; O'Donnell *et al.*, 1979). However, it is not well understood what properties of tissue collagen affect the ultrasonic propagation properties. There is some evidence to suggest that it is the elastic properties of soft tissue, determined primarily by the content of collagen and other structural proteins, which define acoustic contrast during echographic visualization. The hypothesis is based on the fact that the static or low-frequency elastic modulus of collagenous fibers is at least 1000 times greater than that of soft tissues such as smooth muscle which has a very low concentration of collagen (Fields and Dunn, 1973). Tissues with higher collagen concentration such as cartilage and bone exhibit ultrasonic velocity and attenuation coefficients greater than those of soft connective tissues (O'Brien, 1977; O'Donnell *et al.*, 1979). Recently, direct ultrasonic measurements of the speed of sound in mouse tail fiber bundles further supported the idea that clinical echographic visualizability of tissue depends, to some degree, on both the collagen content and its three dimensional arrangement within a particular tissue (Goss and O'Brien, 1979).

The acoustic microscope is used in the present study to determine the ultrasonic propagation properties of dog skin wounds of 7, 10, 14, 21, and 35 days duration. Incisional skin wounds provide a well-characterized, dynamic biological model for investigating the relationships between collagen and ultrasonic propagation properties. The interesting feature of this model is that it utilizes the dynamic biological process of wound healing, in which changes in total collagen content occur as a function of time and changes in collagen configuration occur with wound maturation and remodeling (Madden and Peacock, 1971; Forrester, 1973). Hence it provides the opportunity to study the changes in ultrasonic propagation properties as a function of changes in collagen content and configuration.

I. METHOD

A. Acoustic microscopy

A scanning laser acoustic microscope (Sonomicroscope 100^R, Sonoscan, Inc., Bensenville, IL 60106), operating at a frequency of 100 MHz was employed for direct optical and acoustical examination of wound specimens, as well as the quantitative determination of the speed of sound. The operational details of this instrument can be found elsewhere (Gross and O'Brien, 1979) and are only summarized here. The specimen is placed on a sonically activated fused silica stage and is covered with a mirrored coverslip. Mechanical perturbations of the coverslip surface due to the acoustic energy transmitted through the specimen are detected by a focused, scanning laser beam probe. These disturbances are proportional to the acoustic amplitude in each region. The laser light transmitted through the coverslip and specimen allows the formation of an optical image in perfect register electronically with the acoustic image. The quality of this optical image is quite different from that of a standard optical microscope owing to the laser energy source and to the scanning nature of the system.

The acoustic microscope also operates in an interference mode. Here, an electronic reference signal is added to the acoustic image signal allowing for determination of the change in the phase of the acoustic

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signal passing through the specimen. The interference mode provides the information necessary to determine, at the microscopic level of structure, variations in the velocity of propagation of acoustic energy through the specimen under investigation.

B. Specimen preparation

Seven paravertebral incisions 3 cm in length and 2 cm apart were produced on each side of the back of a 24-kgm mongrel dog. The wounds were incised to the depth of subcutaneous fat after the sites had been aseptically prepared and shaved. Each wound was closed with interrupted 4–0 sutures. All wounds healed per prinum with infection. The healing process for the specimens used in this portion of the study was sequentially disrupted at days 7, 10, 14, 21, and 35 by removing the wound area as a part of an elliptical excision. Sutures were removed and the wound area was reshaved prior to excision of the wound tissue in order to decrease the effect of hair in the subsequent measures. The samples were trimmed of excess fat and a 1-cm strip was cut perpendicular to the axis of the wound, placed in aluminum foil, frozen to the temperature of liquid nitrogen within 5 min of the time of its excision, coded so that they could be evaluated acoustically in a blind fashion and stored at −60 °C in a Revco freezer in vapor tight vials. They were shipped on dry ice from the University of Washington to the Bioacoustic Research Laboratory at the University of Illinois, where they remained frozen at −40 °C until they were prepared for examination for acoustic microscopy. At the time of examination, the coded specimens were thawed to room temperature and cut in half, perpendicular to the wound plane. Each half was trimmed, frozen in an alcohol and dry ice mixture, mounted to the object disk with embedding medium in a manner enabling sectioning in two orthogonal planes, and sectioned with a Lipshaw cryostat microtome. One-half of the specimen yielded sections parallel to the skin surface, while the other half yielded sections perpendicular to the skin surface. As each individual section was cut, it was placed on the acoustic microscope stage and bathed in isotonic saline which served as a coupling medium. Acoustic microscopic examination was performed immediately at room temperature (22 °C) before the next section was cut. Typically, 5 sections ranging in thickness from 30 to 100 μm were examined. Following acoustic microscopic examination, the specimens were stained with hematoxylin and eosin to provide additional optical evaluation of the tissue. Sections were mounted on an albuminized glass slides and stained by Harris' hematoxylin and eosin procedure for post-fixed fresh frozen tissue sections without initially dehydrating the mounted section (Thompson, 1966).

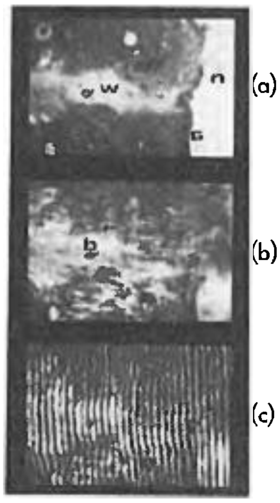

FIG. 2. Optical (a), acoustical (b), and interferometric (c) images of a 50-μm-thick section in isotonic saline n of dog skin wound of 10 days duration cut perpendicular to the skin margin s and wound plane w. The spherically opaque object in the wound plane of all three images is an air bubble b. The typical horizontal distance between vertical interference lines is 85 μm.

II. RESULTS

Figure 1 shows optic, acoustic, and interferometric micrograph montages of a 7-day-old wound section cut 40 μm thick and perpendicular to the skin surface and the wound plane. The wound development was insufficient to provide the necessary mechanical strength, and separation of the tissue margins occurred in this wound. The wound tissue seen along the left margin of Fig. 1(a) is clearly outlined by saline. The interface with normal tissue on the right is less distinct. The wound tissue appears acoustically quite homogeneous and, qualitatively, exhibits acoustic attenuation similar to that of the surrounding saline medium [Fig. 1(b)]. The tissue adjacent to the wound area, however, is acoustically heterogeneous as evidenced by variation in acoustic attenuation. Note that the hair follicles and

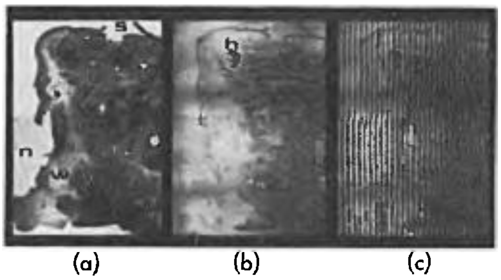

FIG. 1. Optical (a), acoustical (b), and interferometric (c) images of a 40-μm-thick section in isotonic saline n of dog skin wound of 7 days duration cut perpendicular to the skin surface s and wound plane w. Hair follicle is denoted by h. The typical horizontal distance between vertical interference lines is 85 μm.

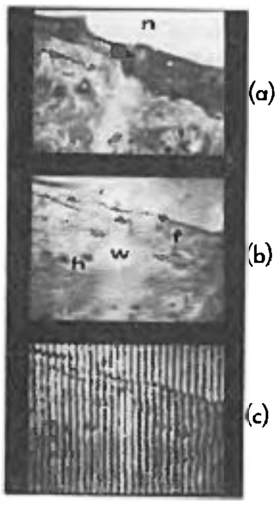

FIG. 3. Optical (a), acoustical (b), and interferometric (c) images of a 30-μm-thick section in isotonic saline n of dog skin wound of 14 days duration cut parallel to the skin surface and perpendicular to the wound plane w. Hair follicles h are noted on the left-side of the wound plane. The upper right part of the specimen is folded over onto itself f. The typical horizontal distance between vertical interference lines is 85 μm.

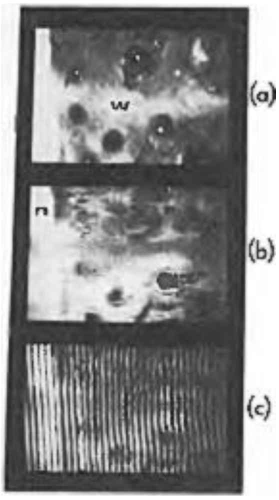

FIG. 4. Optical (a), acoustical (b), and interferometric (c) images of a 60-μm-thick section in isotonic saline n of dog skin wound of 21 days duration cut parallel to the skin surface and perpendicular to the wound plane w. The typical horizontal distance between vertical interference lines is 85 μm.

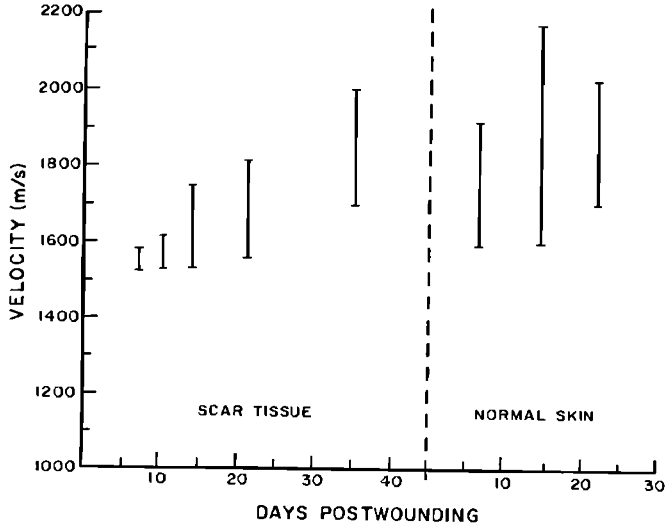

FIG. 6. Range of ultrasound velocities for scar tissue and normal dog skin as a function of wound age. Bars represent maximum and minimum velocities and are not statistically derived confidence limits.

shafts are both optically and acoustically opaque, whereas the 7-day-old granulation tissue is virtually transparent acoustically. The interferogram [Fig. 1(c)] adds further support to the homgeneous nature of the material along the wound margin wherein little variation of acoustic velocity is seen. In contrast, the adjacent normal tissue is quite heterogeneous as noted by the wavy nature of the vertical interference lines.

Preliminary studies suggest that orientation of these wound specimens, relative to the direction of sound wave, does not appreciably affect the acoustical properties. Figure 2 depicts a 50-μm, 10-day-old wound section cut perpendicular to the skin surface and the wound plane. Here, the wound is easily distinguished in both the optic and acoustic micrographs. The wound area delineated optically [Fig. 2(a)] would seem to be smaller than that outlined acoustically [Fig. 2(b)]. A 14-day-old wound seen in Fig. 3(b) from a 30-μm-thick section cut parallel to the skin surface appears to have less contrast to the surrounding normal tissue than the perpendicular sections seen in Fig. 2(b). However, a 30-μm section from the same 14-day-old wound cut perpendicular to the skin surface showed wound acoustic contrast similar to that seen in Fig. 3(b) are attributed to differences in thickness of the sections rather than differences in the orientation of these specimens. For an older wound of 21 days duration, a 60-μm section is cut parallel to the skin surface (Fig. 4). In this thicker section, a decrease in acoustical contrast between the wound area and the surrounding normal skin is again noted. Here, the decreased contrast is attributed to an actual increase in ultrasonic attenuation by the wound tissue, since the acoustical contrast is normally enhanced by thicker section. This observation underscores the importance of examining multiple sections of varying thickness.

The acoustic micrographs and interferograms shown in Figs. 2, 3, and 4 demonstrate the wound area to be acoustically more homogeneous in attenuation and velocity than the adjacent tissue areas. There is also a qualitative trend that is not depicted clearly in these figures, viz., as the wound ages, the acoustic contrast decreases between the wound area and the adjacent normal tissue. This trend is further illustrated by examining a 40-μm-thick, 35-day-old wound section cut parallel to the skin surface (Fig. 5). Here, the wound

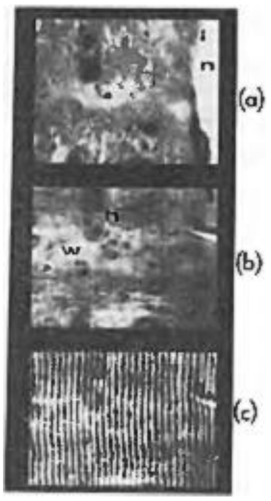

FIG. 5. Optical (a), acoustical (b), and interferometric (c) images of a 40-μm-thick section in isotonic saline n of dog skin wound of 35 days duration cut parallel to the skin surface and perpendicular to the wound plane w. An artifact is shown on the right side of the acoustical and interferometric images and hair follicles are noted by h. The typical horizontal distance between vertical interference lines is 85 μm.

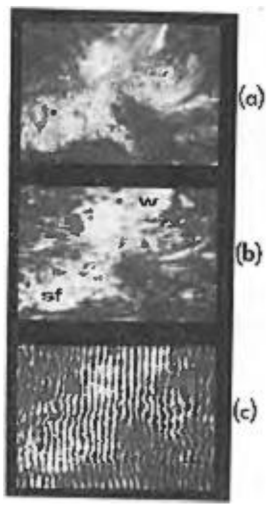

FIG. 7. Optical (a), acoustical (b), and interferometric (c) images of a 50-μm-thick section of dog skin wound of 10 days duration cut perpendicular to the skin surface and the wound margin w. Subcutaneous fat sf is below and adjacent to the wound margin. The typical horizontal distance between vertical interference lines is 85 μm.

TABLE I. The range of ultrasonic velocities for dog wound tissue as a function of age from 7 to 35 days.

Wound age (days)	Velocity range (m/s)	Sample thickness (μm)
7	1540–1575	40
10	1530–1620	50
14	1540–1750	30
21	1560–1820	60
35	1700–2000	40

area and the adjacent tissue appear acoustically quite similar. This observation strongly suggests an increase in acoustic attenuation as wound tissue matures.

Quantitative changes in the acoustical properties of the wound tissue were documented for wounds of 7, 10, 14, 21, and 35 days duration. Table I lists the extremes of the velocity range for each sample (i.e., 100% of all measured values fall within these limits). This is represented graphically in Fig. 6 and shows that the velocity of sound in these samples increases as a function of wound age. Results were not expressed in terms of mean velocity and standard deviation because only maximum and minimum velocity were measured with the SLAM. One would have to assume a normal distribution of velocities between the maximum and minimum in order to report the mean velocity and standard deviation. Presently, the distribution of velocities between the maximum and minimum cannot be measured accurately with the SLAM for heterogeneous material.

Within the normal tissue, the wavy nature of the interference lines can be viewed as representing spatial variations of acoustic velocity. The range of minimum and maximum velocities have been determined for normal skin adjacent to the 7-, 14-, and 21-day-old wounds, and the velocities are listed in Table II. The maximum and minimum velocity parameters of wound tissue and normal dog skin, which are listed in Tables I and II and shown in Fig. 6, appear to encompass a wide range of velocities. These ranges appear large because maximum values for potential sources of error were assumed in making those calculations of velocity. These sources of error include the measurement of the thickness of sections and the measurement of shifts in the interference lines.

Figure 7 shows an area of subcutaneous fat adjacent to a 10-day-old wound from a 50-μm-thick section cut perpendicular to the skin surface and wound plane. Acoustically, the fat appears more heterogeneous than the wound area and less heterogeneous than the surrounding normal tissue [Fig. 7(b)]. The interference lines relative to the wound area shift markedly to the left indicating a lower velocity in the fat. The ultrasonic velocity in the subcutaneous fat ranges from 350 to 600 m/s lower than that in the adjacent wound area and has a range in velocity from 975 to 1225 m/s.

III. DISCUSSION

Madden and Peacock (1971) have shown in rats that wound collagen increases rapidly for about the first three weeks in a healing incised wound. After the third week, the collagen content in the wound stabilizes but the dynamic process continues with new collagen synthesis and removal occurring at the same rate. These observations were related to the data of Levenson *et al*. (1965) which showed that wound strength paralleled the collagen concentration up to about three weeks, at which time wounds have gained less than 20% of their ultimate strength. Thus the balance of wound strength is developed with the wound collagen content stable suggesting that changes of the wound collagen configuration is responsible for the increased strength. The changes in configuration of collagen with wound maturation have been previously documented by standard light microscopy (Levenson *et al.*, 1965) and by electron microscopy (Forrester, 1973).

This initial study of the acoustical propagation properties of maturing wound tissue indicates that there is an increase in the speed of sound and attenuation coincident with an increase in the age of scar tissue. It is suggested that as the wound age increases, the general trend of increasing velocity is related to the increase in collagen content and to the change in the three-dimensional configuration of wound collagen. This is supported, in part, by the fact that the ultrasonic velocity has been shown to increase with increasing collagen

TABLE II. The range of maximum and minimum ultrasonic velocities for normal dog skin adjacent to wound area.

Range of minimum velocity (m/s)	Range of maximum velocity (m/s)	Sample thickness (μm)
1590–1700	1690–1920	30–50
1600–1750	1870–2170	30–40
1710–1950	1750–2030	50–70

concentration in a variety of tissues (O'Brien, 1977; Johnston *et al*., 1979). A similar trend has been shown in which the ultrasonic attenuation, in the low megahertz frequency range, increases with increasing collagen concentration (Johnston *et al*., 1979; O'Donnell *et al*., 1979).

Another important observation from this preliminary study is the very low velocity of sound in subcutaneous fat which exhibits the lowest ultrasonic velocity of any tissue previously measured with the exception of lung tissue (Dunn, 1974). This mismatch of velocity between subcutaneous fat and the normal dermis or wound tissue could provide an excellent interface from which to measure reflective signals for *in vivo* measurements.

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