Studies on human uterine cervix and rat uterus using S-, X- and Q-band electron-spin-resonance spectroscopy

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1. In previous studies we have reported on the detection of a strong e.s.r. signal in samples of normal human cervix; the signal is much reduced or absent in samples of invasive cancer of the cervix. In order to identify the species responsible for the strong signal, we have used X-, S- and Q-band e.s.r. spectroscopy. 2. The major signal that is detectable in ground-up samples of cervix preserved at -196°C has features consistent with the presence of a peroxy free radical. Good agreement with the experimental findings was obtained by computer simulation, using values for the g -tensor of $g_x = 2.002$, $g_y = 2.005$ and $g_z = 2.036$. 3. The peroxy radical is produced on grinding the normal cervix samples to a powder under liquid N_2 , and appears to be formed by modification of a pre-existing oxygen-containing complex. Control experiments eliminated the possibility that the strong signals seen in frozen powders prepared from normal cervix were artefacts only of the grinding procedure. 4. Experiments with rats *in vivo* and with cervix samples *in vitro* are consistent with the conclusion that the peroxy radical is formed by disturbing the cyclo-oxygenase system that is involved in prostaglandin synthesis.

A strong free-radical signal has been detected in frozen powders of normal human cervix using X-band e.s.r. spectroscopy; the signal was markedly reduced in corresponding samples obtained from patients with invasive cancer of the cervix (Slater & Cook, 1970). Recent experiments have confirmed the original observations mentioned above, and have shown that the main signal located close to the free-electron-spin position, and an associated minor signal located at $g = 2.03$, are not easily saturated with increasing microwave power (Benedetto *et al.*, 1981). The signals in frozen powders of human cervix were sensitive to an increase in temperature, and the signals were not restored on recooling the samples; it was suggested that the signals were due to the occurrence of a lipid peroxy free-radical species in the frozen powders (Benedetto *et al.*, 1981).

The objective of the present study was to identify the free-radical species responsible for the unusu-

ally strong free-radical signals observed in frozen powders of normal cervix. The evaluation of those metabolic disturbances occurring in carcinoma of the cervix that result in a decrease of the e.s.r. signal will be aided considerably by identification of the free-radical species involved. To achieve our objective we have used e.s.r. spectroscopy at three different frequencies (S-, X- and Q-band), and also have performed experiments with normal rat uteri to test the effects of metabolic inhibitors *in vivo* as well as *in vitro*.

Experimental

Samples of human cervix were obtained at operations for hysterectomy, which were performed for reasons not involving pathological disturbance of the cervix. The samples were selected to contain the region of the squamo-columnar junction, and were cut into small pieces (approx. $10\text{ mm} \times 3\text{ mm} \times 3\text{ mm}$) and immediately frozen in liquid N_2 . The frozen samples were

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subsequently ground to a powder under liquid N₂ as described previously (Benedetto *et al.*, 1981).

The rats used were sexually mature females of the Wistar strain (body wt. approx. 300 g). The rats were killed by cervical dislocation, and samples of the uterus were taken to include a short length of the upper vagina, the cervix, and short lengths of the lower uterine horns. The samples were frozen and powdered as for human samples. Some rats received one intraperitoneal injection of indomethacin (10 mg/kg body wt. in 0.5 ml of 0.9% saline) 3 days before they were killed; control rats received a similar volume of saline.

Some samples of human cervix were incubated for 10 min at room temperature with antioxidants and other substances (see below) before being frozen and ground under liquid N₂. DL- α -Tocopherol was obtained from ICN Pharmaceuticals, Cleveland, OH, U.S.A.; vitamin C (ascorbic acid) and indomethacin from Sigma Chemical Co., St. Louis, MO, U.S.A.; butylated hydroxyanisole was from BDH Chemicals, Poole, Dorset, U.K.

A Varian E-109 e.s.r. spectrometer equipped with an E-102 bridge was used for experiments in the X-band region (9.1 GHz). An E-110 bridge was used for experiments in the Q-band region (35 GHz). Experiments using S-band (2–4 GHz) facilities were done at the Medical College of Wisconsin, National Biomedical e.s.r. Centre, Milwaukee, WI, U.S.A., with the helpful co-operation of the Director, Dr. J. Hyde; these experiments used a microwave bridge equipped with a loop-gap resonator as described by Fronciz & Hyde (1982). The loop gap resonator has a higher filling factor, and a smaller size than the conventional TE-102 cavity for S-band studies, and thus gives a higher sensitivity for samples of limited size. The temperature of the cavity in e.s.r. experiments was regulated with a Varian variable temperature controller.

The e.s.r. spectral data were collected and averaged by using a Varian 620-i computer with CLASS language (Klopfenstein *et al.*, 1972).

Results

Samples of normal human cervix contain a high concentration of a free radical(s) that can be detected by e.s.r.; the signal is in the range $g = 2.11$ – 2.15 for intact frozen samples, and close to $g = 2.00$ for frozen powders of cervix (see Benedetto *et al.*, 1981). The signal observed in frozen powders is very sensitive to increases in temperature (unlike the case with frozen intact samples), and irreversibly disappears when the temperature is raised above about -70°C (Benedetto *et al.*, 1981). The results given below refer

generally to work with frozen powders, except where the use of intact samples is emphasized.

If the major features of the X-band response (Fig. 1*b*) are due to anisotropy, then the components of the g -tensor will vary in their relative separation according to the microwave frequency. Conversely, if the major features represent hyperfine splitting, then no significant change in relative separation would be seen on varying the frequency.

X-, S-, and Q-band e.s.r. spectroscopy show (Fig. 1) that the signal at high field represents the $g_{\perp}$ component of the g -tensor whilst the low-field

Fig. 1. S-, X- and Q-band e.s.r. spectra of powdered samples of normal human cervix

Instrument settings: S-band: field setting, 0.1185 T (1185 G), 3.28 GHz; modulation amplitude 0.5 mT (5 G); gain $10^4 \times 3.2$ (attenuation 10 dB); microwave power 20 mW; temperature -150°C ; field scan 20 mT (200 G). X-band: field setting, 0.3250 T (3250 G), 9.14 GHz; modulation amplitude 0.5 mT (5 G); gain $10^3 \times 5$, microwave power 20 mW; temperature -150°C ; field scan 20 mT (200 G). Q-band: field setting, 1.2140 T (12140 G), 35.4 GHz; modulation amplitude 0.5 mT (5 G); gain $10^3 \times 5$; microwave power 25 mW; temperature, -150°C ; field scan 40 mT (400 G). This spectrum is the average of ten scans with background subtraction (see Fig. 2).

feature is due to the $g_{\parallel}$ -component. With S-band frequency the two features come together, whereas with Q-band the features have separated further. The weak intensity of the low-field feature under Q-band conditions necessitated the use of signal averaging and subtraction of the cavity background signal (Fig. 2).

It can be seen from Fig. 2(c) that, in addition to the separation of $g_{\perp}$ and $g_{\parallel}$ in the Q-band study, there is an additional splitting of the perpendicular feature, indicating that the symmetry of the free radical under study is not completely axial. Computer simulation (Fig. 3c) of the Q-band spectrum suggests that the splitting of the $g_{\perp}$ -component arises from a rhombic splitting and not from the presence of another free radical. However, the major feature seen at S-, X- and Q-band frequencies probably contains more than one free-radical species, since, at microwave powers of less than 5 mW, the ratio of $g_{\parallel}$ to $g_{\perp}$ is not constant (Fig. 4 for X-band). This point is illustrated in

Fig. 2. Q-band spectra of powdered samples of normal human cervix

Each signal (a, b and c) is the result of ten averaged spectra. (a) Background signal; (b) sample signal; (c) result of the subtraction (b-a). Experimental settings: field setting, 1.2140 T (12140 G), 35.4 GHz; modulation amplitude 0.5 mT (5 G); gain $10^3 \times 5$; microwave power 25 mW; temperature -150°C ; field scan 40 mT (400 G).

more detail in Fig. 5 for both X-band and Q-band studies. It can be seen that the major feature present is not saturated over the power range studied, whereas the 'hidden' species saturates readily.

Some samples of normal human cervix were ground in liquid N_2 in an N_2 atmosphere, after having first subjected intact samples to a freeze-thaw cycle under vacuum to decrease the content of free dissolved O_2 ; no significant change was seen in the X-band e.s.r. signals in samples so

Fig. 3. Spectra produced by a computer simulation of the S-, X- and Q-band spectra illustrated in Fig. 1

The linewidths increase with increasing frequency of the microwave radiation, and with increasing shift from $g = 2.00$. The lineshapes are approximately Gaussian. The parameters used in calculating the spectra are: $g_x = 2.002$, $g_y = 2.005$, $g_z = 2.036$. S-band: field setting, 0.1185 T (1185 G), 3.2791 GHz; field scan 20 mT (200 G); linewidth (measured at half-width, half-height): $w_x = 0.57$ mT (5.7 G), $w_y = 0.57$ mT (5.7 G), $w_z = 0.57$ mT (5.7 G). X-band: field setting, 0.3250 T (3250 G), 9.14 GHz; field scan 20 mT (200 G); linewidth: $w_x = 0.71$ mT (7.1 G), $w_y = 0.71$ mT (7.1 G), $w_z = 1$ mT (10.0 G). Q-band: field setting, 1.2140 T (12140 G), 34.4 GHz; field scan 50 mT (500 G); linewidth: $w_x = 0.79$ mT (7.9 G), $w_y = 0.9$ mT (9.0 G), $w_z = 3.54$ mT (35.4 G).

Fig. 4. Powdered samples of normal human uterine cervix: X-band e.s.r. spectra obtained at (a) 0.1 mW and (b) at 50 mW

Other conditions were as for Fig. 1.

prepared when compared with samples ground (i) under liquid N_2 in air or (ii) after first exposing the intact sample to pure O_2 gas before grinding in liquid N_2 in an O_2 atmosphere. The lack of effect of grinding under reduced or increased oxygen partial pressures indicates that the peroxy species arises from a pre-existing O_2 complex or can be formed maximally under very low O_2 partial pressures.

Some intact samples of normal human cervix were incubated with 2–10 mM concentrations of different antioxidants (vitamin E, vitamin C, butylated hydroxyanisole) for 10 min at $37^\circ C$ before freezing and grinding under liquid N_2 ; no significant changes in the major X-band features were seen, although with vitamin E a characteristic phenoxy-radical spectrum was observed. The formation of the peroxy radical during grinding under liquid N_2 is probably the explanation of the lack of significant effect found on incubating unfrozen intact cervix samples with antioxidants; the interaction of such materials with a lipid peroxy free radical in the frozen powder is expected to be very slow.

As already reported by Benedetto *et al.* (1981), the major signal features in X-band spectra disappear on warming the frozen powder to temperatures higher than approx. $-70^\circ C$; the signals do not reappear on recooling to $-196^\circ C$, with or without regrinding under liquid N_2 ; the main signal in frozen powders of normal cervix is not therefore an artefact of the grinding process.

Fig. 5. Power saturation of the anisotropic e.s.r. signal (O) observed in frozen powders of normal human cervix, and the calculated power saturation of a 'hidden' free-radical species (●) within the $g_{\perp}$ -feature of the main anisotropic signal

It was assumed that the major contribution to the $g_{\perp}$ -feature is the anisotropic response of a peroxy radical. By determining the ratio of the signal intensities of the perpendicular to the parallel features at high microwave power and regarding this as a constant throughout the power range for the anisotropic component, it was possible to obtain an estimate of the contribution of the 'hidden' component by comparing the calculated value with that actually measured at low microwave powers. This estimated contribution from the 'hidden' component is shown as being easily saturated, in contrast with the anisotropic component.

In view of the possible relationship of the e.s.r. signal to the prostaglandin synthetase pathway (Benedetto *et al.*, 1981), some experiments have been done with rat uteri. After the intraperitoneal

Fig. 6. X-band e.s.r. spectra of powdered samples of rat uterine cervix

Experimental conditions were as in Fig. 1. The anisotropic signal at $g = 2.010$ [normal sample, (a)] is significantly reduced in the indomethacin-treated rats (b); moreover, two new intense features at $g = 2.24$ and $g = 4.3$ appear in the samples pretreated with indomethacin.

injection of indomethacin for 3 days before sampling, freezing and grinding, the main e.s.r. feature at X-band frequency was significantly decreased. The e.s.r. signal intensity (in arbitrary units) in normal control samples was 54 ± 6 (seven experiments); the corresponding mean value ($\pm$ S.E.M.) after treatment with indomethacin was 24 ± 5 (seven experiments), $P < 0.005$. Moreover, pretreatment with indomethacin produced large changes in the X-band e.s.r. spectrum such that strong signals appeared at $g = 4.3$ and $g = 2.24$ (Fig. 6).

Discussion

The results presented here, using 2 and 35 GHz e.s.r. spectroscopy, confirm the suggestion made previously (Benedetto *et al.*, 1981) on the basis of the 9 GHz X-band study that the two main signals seen using X-band conditions in powdered frozen samples of normal human cervix are features of the same free-radical species. The linewidth increases with increasing microwave frequency, and this effect is most marked with the low-field g_1 -component (Fig. 3). The line-broadening mechanism is known as g -strain (Fronciz & Hyde, 1980) and is due to small variations in the local site

geometry that results in a distribution of the magnetic parameters.

The symmetry of the major free-radical species in powdered frozen samples of normal human cervix is almost completely axial. The values obtained for the g -tensor are $g_x = 2.002$, $g_y = 2.005$ and $g_z = 2.036$. Such values are typical of organic peroxy free radicals (Chien & Boss, 1967; Kuznetsov & Radstag, 1972). No hyperfine splitting was detectable, even at 3 GHz; the low-frequency S-band studies have the potential to resolve hyperfine structure that is obscured at higher frequencies as a result of the line-broadening effect already noted.

Although no indication of hyperfine splitting was obtained, some clear evidence for the presence of additional free-radical species with isotropic features located close to the free-electron-spin position was found (Figs 4 and 5). The easily saturated free-radical component(s) that are obscured by the strong $g_{\perp}$ -component of normal human cervix can be assigned to the various organic radicals that are a common feature of many different types of biological materials (see Swartz, 1972).

It is known (Benedetto *et al.*, 1981) that frozen intact cervix gives a strong X-band signal in the region of $g = 2.11$ – 2.15 , and that this is decreased on grinding under liquid N_2 to yield the signals discussed here with g -values around 2.0–2.03. Although the grinding process itself undoubtedly changes the free-radical components present in the tissue sample, it appears to do so largely by an interconversion of the species with the higher g -value to the species with a lower $g_{\perp}$ -value rather than to generate a completely new and quite 'artefactual' free radical (Benedetto *et al.*, 1981). That conclusion is strengthened by our present finding that regrinding and recooling a frozen powder that had previously been allowed to warm up did not produce any significant e.s.r. signal.

Indomethacin is a well-known inhibitor of prostaglandin synthetase (see Vane, 1978) and treatment of rats *in vivo* with this compound significantly decreased the strength of the e.s.r. signal observed in frozen powders. These observations are suggestive evidence that the parent metabolic process being uncovered by these e.s.r. studies is the cyclo-oxygenase-dependent prostaglandin cascade. Certainly, cervix and endometrium are known to be very active metabolically in that respect (Ellwood *et al.*, 1980; Massobrio *et al.*, 1981).

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