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ELECTRON SPIN RESONANCE STUDIES OF NITROSYL HAEMOGLOBIN IN HUMAN LIVER, COLON AND STOMACH TUMOUR TISSUES

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Iron nitrosyl haemoglobin (HbFeNO) gives well defined ESR spectra, and can be detected at room temperature, in contrast with most transition metal complexes of biological importance. This is because the unpaired electron remains strongly localised on the NO ligand. It is of importance because it proves the formation of nitric oxide, which unfortunately cannot be detected directly by ESR spectroscopy. We have studied a range of tissues taken from human liver, colon and stomach tumours which have been directly frozen to 77K and studied at 77K. The results show that formation of HbFeNO is rare in tissue adjacent to tumour tissue ("peripheral tissue"), but is always found in necrotic central regions, if present. However, in several cases, HbFeNO was also detected in tumour tissue which was not necrotic. Two factors contribute to the formation of this complex. One is the presence of "free" NO molecules in the cellular regions, and the other is the presence of deoxyferrohaemoglobin, since neither ferrihaemoglobin nor oxyhaemoglobin react to give this complex. [For systems containing myoglobin these comments include the possibility of the formation of nitrosylmyoglobin, which gives very similar ESR spectra.]

KEY WORDS: ESR/nitroxide/nitrosyl haemoglobin/human tumours.

INTRODUCTION

In early ESR spectroscopic studies of tumour tissues a strong feature in the free spin ($g = 2$) region was frequently observed that was originally ascribed to free radicals.[1] This assignment was accepted because of a general expectation at that time that radicals were in some way responsible for tumour development and growth. However Maruyama *et al.*, eventually showed that this signal was due to the presence of iron-nitrosyl derivatives ($Fe^{II}NO$), rather than to specific tumour related free radicals.[2] This has been fully corroborated by others.[3] Furthermore, since this signal seemed to be observed in most tissue samples, rather than being confined to tumours, interest was generally lost.

It is now recognised that nitric oxide (·NO), or a derivative of ·NO that acts as a ready source thereof, is of extreme importance in a variety of cellular functions.[4] In principle, since ·NO is a doublet state radical, ESR spectroscopy should be a useful tool in detecting its formation and estimating concentrations. This is especially the case since the ESR method can be used on all forms of freshly obtained tissue

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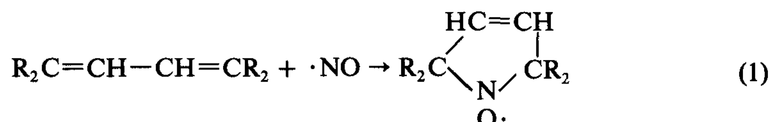
samples without the need for lengthy manipulation. Thus, the samples can be quickly frozen to 77K and the ESR spectra studied at that temperature. Nothing should interfere with the measurements unless other paramagnetic species are present giving overlapping lines, which is unusual.

Unfortunately, because of orbital degeneracy and the resulting orbital magnetism, the ESR spectra for ·NO even in such frozen samples is usually too broad for detection. Also, the normal techniques of spin-trapping, that convert reactive free radicals into very stable and readily detectable nitroxide radicals, fail for ·NO because of its low reactivity. However, a reaction of dienes with ·NO to give stable nitroxide radicals has recently been discovered.[5,6] An example is given in reaction (1).[6]



(1)

Such reactions may well become important in the detection of ·NO, but require the presence of the trapping agents in the region of interest, and that such agents be non-toxic.

Transition metal complexes are also good traps for ·NO, and deoxymyoglobin or deoxyhaemoglobin are amongst the most efficient. The great advantage of these traps is that they are present in certain natural tissues. The association constants are large, being comparable with those for dioxygen. Thus, in any system containing these proteins, ·NO will be trapped, and can be estimated using ESR spectroscopy at 77K. Conversely, if a tissue, sample shows these characteristic ESR features, then the presence of both ·NO, and deoxyhaemoglobin (or deoxymyoglobin) is established. Once formed, the complex is stable for several hours even in the presence of oxygen.

In our present study, we have examined a range of human tumours using ESR spectroscopy, in a search for the conditions under which HbFeNO centres tend to accumulate. [The ESR parameters for HbFeNO vary slightly, depending on the form of the globin protein (relaxed or tense), and on the pH.[7] The spectra that we have obtained have probably been mixtures, but are clearly identifiable as the nitrosyl derivative, with three well defined ^{14}N components as in Fig. 1]. Samples were taken from surrounding healthy tissue, 'peripheral' tissue, and tumour tissue. Contrary to previous findings we generally failed to detect the ESR spectrum of HbFeNO in the surrounding or peripheral tissues, and only in certain tumour samples (Table 1). In all cases in which there were necrotic regions, this signal was obtained, but that is not exclusive. In some cases, clear iron-nitrosyl signals were observed from non-necrotic tissue.

MATERIALS AND METHODS

Sample Preparation

Tissue samples were prepared as soon as possible after surgical excision. This time was generally less than 5 minutes. The tissue was then divided to provide at least three samples: central tumour, peripheral and normal (undiseased) tissue. Each of these was then shaped into a 15 × 3mm cylinder with non-ferrous instruments and rapidly frozen in liquid nitrogen. All samples remained at 77K until after initial ESR investigation.

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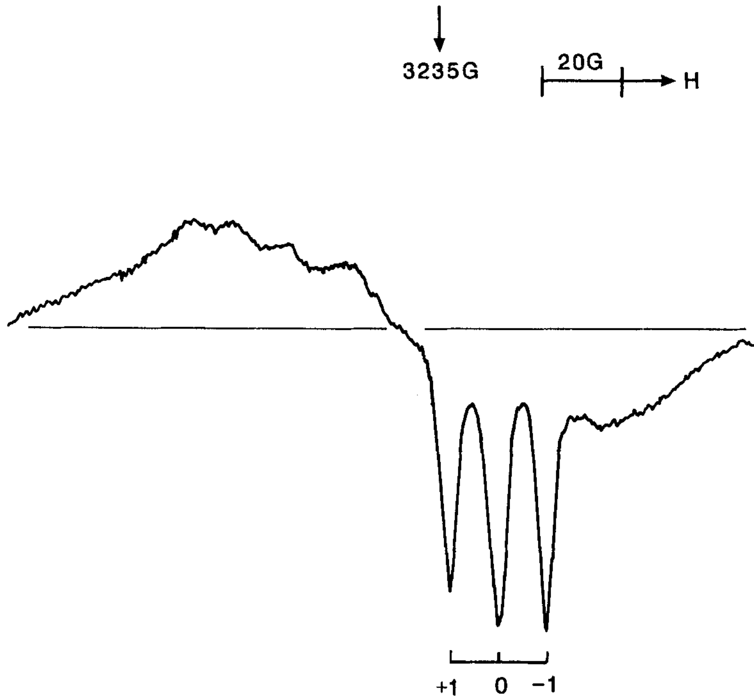

FIGURE 1 First derivative X-band ESR spectrum for nitrosyl haemoglobin taken from the necrotic region of a stomach tumour, and measured at 77K using 2G modulation and a microwave power of 20mw. It is typical of such powder spectra that only the central features are well defined.

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TABLE I
Details of the ESR spectra of various human tumour samples

Sample	$g = 4.3$/mm	$g = 6$/mm	FeNO/mm
Liver (PM) tumour	0.96	0.0	0.32
Liver (PM) normal	0.93	0.0	0.0
Liver tumour (PM)	0.81	0.0	0.0
Liver normal (PM)	0.41	0.0	0.18
Colon tumour	0.11	0.0	0.39
Colon normal	0.31	0.48	0.0
Colon tumour	0.42	0.0	0.0
Colon tumour	2.77	0.0	0.37
Colon peripheral	0.20	0.0	0.0
Stomach tumour (NEC)	2.04	0.81	0.95
Stomach tumour (viable)	0.46	0.0	0.00
Stomach normal	0.46	0.0	0.0

PM = post mortem
NEC = necrotic
mm = intensity of the ESR feature in mm under identical conditions, giving relative concentrations.

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ESR Spectroscopy

X-band ESR spectra were recorded on a Varian E-109 spectrometer. Spectra were recorded using quartz finger Dewars with the sample under liquid nitrogen. Typical spectrometer settings are indicated in the caption to Fig. 1.

Histopathology

All the tumours studied were confirmed histologically as adenocarcinomas showing typical microscopic features for their site of origin. The presence or absence of zones of necrosis was recorded in each case.

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RESULTS AND DISCUSSION

The most significant aspect of these results is the clear absence, for most samples, of ESR signals from FeNO derivatives, rather than their presence. Most of the previous literature on tissue samples has stressed the ubiquitous presence of these species and hence their importance has, perhaps, been overlooked. We stress that two factors are required before there can be detectable accumulation of HbFeNO, which is generally the major source of these ESR signals. One is that there must be some degree of hypoxia: in the presence of 'normal' oxygen concentrations ·NO reacts with oxygen, and with oxyhaemoglobin to give ferrihaemoglobin. Displacement of O_2 by ·NO from oxyhaemoglobin does not normally occur, so far as we know. The other requirement is a nearby source of ·NO. Under these conditions, reaction (2) may proceed, thereby trapping the ·NO radical, making it unambiguously detectable, and relatively long-lived.

$$HbFe^{II} + \cdot NO \rightarrow HbFe{-}NO \quad \ldots\ldots (2)$$

When normal, oxic tissue samples are frozen to 77K directly after excision, reaction (2) will not have occurred because of the extremely low concentration of both reactants. Similarly, when oxic tumour samples are frozen, there should be no signal. Thus our negative results are as expected. It seems probable that the positive results reported in the early literature arose primarily because the tissue samples were not frozen directly after excision, and preliminary studies strongly support this suggestion. Some typical results are given in Table 1, and a typical spectrum is shown in Fig. 1.

In all cases in which necrotic tissue was found in the tumour samples, there were high concentrations of HbFeNO. This accords with work on murine tumours, which were usually well developed and had extensive necrosis.[3,8] However, the reverse is not the case. There were several tumour samples that gave clear HbFeNO signals in the absence of detectable necrosis. These would appear to be the key results. They demonstrate that, prior to the onset of necrosis, there may be hypoxic regions in which NO radicals are extensively generated. The reason for hypoxia is presumably poor and weak vasculature. One reason for ·NO generation may simply be that some cell membranes become disrupted such that Ca^{2+} ions can enter, thereby triggering ·NO synthesis via the calmodulin-mediated ·NO synthase, (see for example, ref. 9). If this applies to red blood cells, containing $HbFe^{II}$, then reaction (1) would occur with high probability. However we do not rule out the possibility of an 'immunological' source. Areas of inflammation can often be detected around some

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tumours, and nitric oxide is thought to be one of the active agents involved. Once trapped, the nitrosyl haemoglobin derivative is not thought to be a reservoir of nitric oxide. Following tissue reoxygenation, HbFeNO will react to form nitrite ions which possess neglible activity.

There are, undoubtedly other possible explanations for the formation of detectable quantities of HbFeNO complexes. We hope that the topic is of sufficient interest to generate more work in this area. We stress that our studies of murine tumours, for which HbFeNO was almost invariably detected, show that it is far more informative to work with human tumours when possible.

Other Paramagnetic Centres

During the course of our studies, other paramagnetic species were identified and all the combined information available from ESR spectroscopy will constitute a separate report. The major species always detected was transferrin with a "g - value" of 4.3 (this is not a true g-value, but simply a 'field-marker'). There is a second centre in this region found only within the tumours, which is at present unidentified. The other major centre is ferrihaemoglobin with a strong ESR feature at $g = 6$. This was mainly observed within the tumour, being particularly intense in breast tumours. Two possible causes could be a local degradation of the vasculature, or a major generation of ·NO which reacts with oxyhaemoglobin in fully oxygenated systems to give the ferric derivative.

The other major centre was Cu(II). However, this was rarely observed for the rapidly frozen samples, but grew in when the samples were allowed to stand at room temperature.

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Accepted by Professor J.M.C. Gutteridge

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