A Free Radical in Human Cerebrospinal Fluid Investigated by EPR

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We have detected a free radical in human cerebrospinal fluid (CSF) and have identified as ascorbyl radical using electron paramagnetic resonance (EPR). EPR spectrum of the radical in CSF was compared with one in the corresponding serum. Determination of the ascorbyl radical concentrations in CSF and serum was attempted using known concentrations of a nitroxyl radical. Moreover, an alternative method to measure the biological radical was provided.

Vitamin C (Ascorbic acid, AsA) has been of great interest in relation to human health and disease.[1] It has been suggested that variation of ascorbate concentration closely relates to blood, oxidative stress and other diseases.[2-5] The intrathecal constancy of ascorbyl might be of relevance to antioxidant protection against oxidative degradation processes in diseases. The biological reaction intermediate (ascorbyl radical, AsR) of ascorbic acid in serum for various diseases including blood disease was studied by Sasaki and co-workers.[2] However, there is no evidence of AsR in cerebrospinal fluid (CSF). It has been suggested that AsA reacts with oxygen radicals as a radical scavenger. The reaction can be evidence of a protective homeostatic mechanism against diseases. Investigation of the reaction intermediate in CSF incorporated with serum could provide detailed information about antioxidant reaction processes since ascorbate in serum is diet-dependent. Consideration of CSF and serum will be more adequate because the choroid plexus is responsible for active transport of ascorbic acid from blood to CSF.[3] CSF is also important source of information used to diagnose patients.

We have measured a free radical in CSF using electron paramagnetic resonance (EPR, or ESR)[6] techniques. EPR is one of the best methods to measure biological free radicals directly. The radical can be attributed to the direct involvement of biological processes. We have also introduced an alternative method to measure the radicals.

CSF samples and the corresponding serum samples were directly obtained from acute lymphoblastic leukemia (ALL) patients having no therapy. A commercially available stable free radical, 4-hydroxy-2,2,6,6-tetramethyl piperidinyl-1-oxy (TEMPOL, Aldrich Chemical Co., Milwaukee, Wisconsin, USA) was employed to determine relative concentrations in CSF and serum, and used as received.

Sample solutions directly from the patients were put into poly(methyl methacrylate) capillary tubes (O.D. 1.75 mm, I.D. 0.9 mm, sample volume 30 μl) and sealed at the bottom. The capillary containing the sample was put into a 4 mm EPR tube (O.D. 4 mm, I.D. 2.8 mm) and mounted in the cavity.[7,8] This capillary method allowed us to make systematic measurements easily on a number of samples, instead of using a quartz flat cell. The quartz cell is not efficient for making a number of measurements on biological samples because thoroughly clearing to remove residual contaminants before each measurement is difficult and time consuming. Furthermore, a big advantage of this is inexpensive and disposable capillary after each measurement. In order to confirm the radical species, we performed photo-irradiation of CSF solutions using quartz capillaries (O.D. 1.3 mm, I.D. 0.9 mm).

EPR measurements were made with a JEOL FE 1X X-band spectrometer operating in the TE_{011} mode (cylindrical cavity). The resonance frequency was measured using an EMC-14 X-band microwave frequency counter (Echo Electronics Co., Ltd., Japan). No attempts were made to deoxygenate the sample solutions. All EPR measurements were performed at ambient temperature. CSF and serum samples were measured with a single scan at the same EPR settings throughout.

The EPR spectra of CSF and serum obtained from an ALL patient having no therapy are shown in Figure 1. The EPR spectra for both CSF and serum are a characteristic doublet with a = 0.18 mT and g = 2.005. The hyperfine coupling constant and g-value are identical to those previously reported for ascorbyl

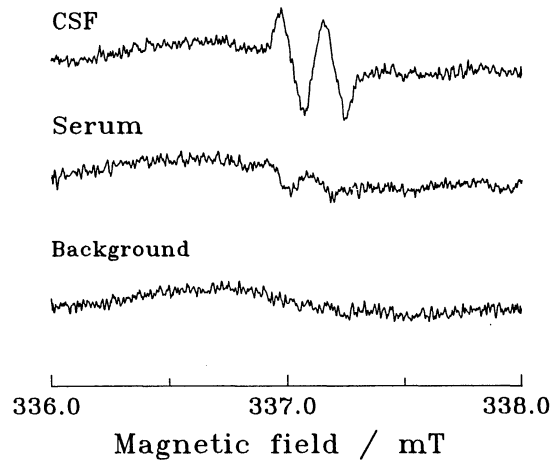

Figure 1. EPR spectra of CSF and serum. The bottom spectrum is background. EPR conditions were the following: microwave frequency, 9.44 GHz; microwave power, 10 mW; modulation amplitude, 0.1 mT; time constant, 0.1 s; sweep rate, 0.25 mT per minute; sweep width, 2 mT.

radical.[9] The signal intensity of the radical for serum is approximately 1/3. The broad background signal around the center is due to the capillary.

We have examined the possibility of the EPR signal coming from either proteins or cells in the CSF sample. The EPR signal obtained from the filtrated solution was identical to that obtained for the unfiltrated CSF measurement. To further ensure the EPR signal is ascorbyl radical from ascorbate in CSF, we have irradiated the sample. UV irradiation of ascorbate can induce the ascorbyl radical.[10] When the CSF sample of Figure 2 (B) was irradiated by UV (500 W xenon arc lamp, long pass filtered at 290 nm) for 20 seconds at the arrow indicated, the EPR signal was enhanced about three times (Figure 2 (C)). Therefore, based on the EPR parameters and the various experimental results, we have assigned the radical as ascorbyl radical.

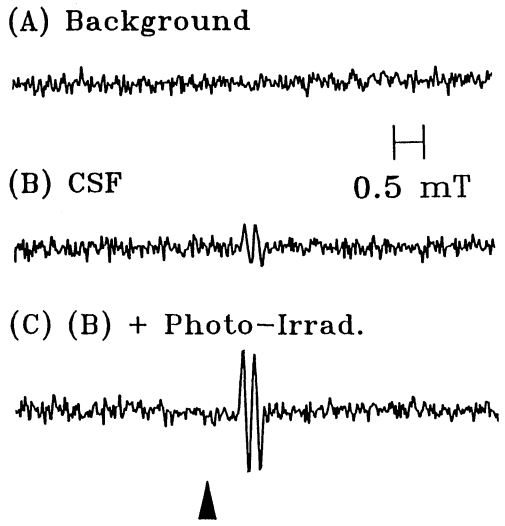

Figure 2. (A) EPR spectrum of background using the quarts capillary. (B) EPR spectrum of CSF obtained from ALL subject age 9, male. (C) EPR spectrum obtained by 20 seconds UV irradiation of (B) at the arrow indicated.

The concentrations of ascorbyl radical in CSF and serum can be determined using known concentrations of the stable free radical, TEMPOL.[11] Fixed concentrations of TEMPOL in pH 7.4 phosphate buffer were measured at the same experimental conditions used for the CSF and serum samples in Figure 1.[12] The total area of TEMPOL were compared with the total area of the radical. The AsR concentrations obtained for CSF and serum (Fig. 1.) were 3.5×10^{-8} mol dm^{-3} and 9×10^{-9} mol dm^{-3}, respectively. The concentrations were obtained using a calibration curve of TEMPOL standard (n = 4). The standard deviation from the straight line is 6×10^{-9} mol dm^{-3}. Further investigations regarding AsR of ALL patients are undergoing.

In conclusions, we found a free radical in the CSF of ALL patients having no treatment and have determined that the species is ascorbyl radical. In order to have reliable results, we have measured CSF samples right after withdrawal from the patients. Also, we have introduced the alternative capillary method to measure AsR in CSF and serum. Although the capillary shows the background signal, this is fast and inexpensive method to measure a number of biological samples. We compared the relative concentrations of the radical in CSF and serum.

Finally, the present EPR studies have provided further information regarding ascorbyl radical in human malaise and demonstrate the potential clinical applications of EPR free radical research.

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References and Notes

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6 ESR is synonymous with EPR. EPR terminology is recommended by IUPAC.
7 This capillary method has been applied for patent. Application number is 5-95213.
8 The capillary method is possible for EPR spectrometer with a cylindrical cavity because of the high sensitivity.
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11 We have used TEMPOL as a standard because it is water soluble and forms a stable radical in aqueous solution.
12 For the calculation of both concentrations, 5 mT sweep width was chosen. All EPR conditions were the same except the sweep width. We took double integrations for all peaks of TEMPOL and the total area was compared with the ones for CSF and serum.