Free Rad. Res. Comms., Vol. 1, No. 6, pp. 387-394
8755-0199/86/0106-0387 $25-00/0

Printed in Great Britain

TREATMENT OF RADIOFIBROSIS WITH LIPOSOMAL SUPEROXIDE DISMUTASE. PRELIMINARY RESULTS OF 50 CASES

F. BAILLET, M. HOUSSET

Service de Radiotherapie
Hopital Necker, Paris, France

A.M. MICHELSON† and K. PUGET

Institut de Biologie Physico-Chimique
13, rue P. et M. Curie, 75005 Paris, France

(Received October 9, 1985)

Well and long established radio-fibroses have been treated successfully with a liposomal encapsulated bovine copper superoxide dismutase. After a short treatment (three weeks intramuscular injection of 5 mg twice a week) regression of the fibrosis is stable. The average size is reduced by one third and significant softening occurs in 82% of the cases. Efficiency is independent of the time between radiotherapy (origin of the fibrosis) and treatment with liposomal SOD. Complete regression even after this limited treatment is seen in cases of chronic prefibrotic inflammatory syndromes and prophylactic action in cases where the probability of fibrosis formation is certain appears to be successful. The roles of superoxide and superoxide dismutase are discussed.

Key words: Liposomal SOD; radioinduced fibrosis; superoxide

INTRODUCTION

The treatment of radio-induced fibrosis is essentially prevention by avoidence. If during radiotherapy an excessive irradiation is employed (for various reasons) a local pathological situation is established which inevitably leads to fibrosis. This phenomenon only occurs above a certain irradiation limit and the time of formation and appearance of the fibrosis is decreased with increase of the radio-dose. Classical treatments such as corticotherapy or use of "cicatrising agents" are relatively inefficient to prevent this evolution, and no treatment exists which causes a well established fibrosis to regress. Although radiotherapeutic techniques have indeed shown a considerable progress with respect to precision of dose levels and of localisation over the past 10 years, in certain situations excessive irradiation is unavoidable, and in any case a non-negligable population of subjects with established post-

† Correspondence and reprint requests to A.M. Michelson.

radiotherapeutic fibrosis exists at the present day. A possible treatment of such radio-induced fibrosis is thus of some interest. In addition, such post-irradiation fibrosis can be considered almost as an experimental human model and successful treatment could serve as a basis, or at least give some indications, for the therapy of fibroses or fibrochondritis arising from other origins.

Use of liposomal superoxide dismutase (LIPSOD) for the treatment of various pathologies such as severe Behçets disease and mucocutaneous lymph node syndrome (Kawasaki disease) has been previously described[1]. Of particular interest was the impressive effect of intramuscular injection of LIPSOD in two cases of terminal stage patients with progressive systemic sclerosis manifesting extensive pulmonary fibrosis[1]. In addition, LIPSOD has been extensively used for the treatment of radio-induced lesions (whether accidental of post radiotherapeutic) with considerable success[2] and in one case 18 years after the original event. It was thus possible that this approach could be useful if applied to solid fibroses, long established or recent, sequels of radiotherapy. This report presents studies of 50 cases, and suggests that such a treatment is of considerable value.

PATIENTS AND METHODS

Fifty patients were included in this initial survey of the efficacity of LIPSOD for the treatment of post-radiotherapeutic fibrosis. With respect to the original cancers, these comprised 30 breast cancer, 15 ORL, 2 prostate, 1 ovary, 1 testicule, 1 penis and 1 malignant melanoma. The elapsed time between therapy of the cancer and treatment with LIPSOD was equal to or greater than 5 years in 11 cases (the two extreme cases were 12 and 17 years), between 2–5 years in 14 subjects and less than 2 years in 25 cases. In this last group, 8 precocious fibroses were treated, 2 prefibrotic inflammatory syndromes, 2 laryngeal edemas, 2 chronic cases of epithelitis, 2 cases of persistant radiomucitis with small necroses and in 1 case to prevent local complications after subsidiary curietherapy. The 8 remaining subjects could not be evaluated due to an interuption of the treatment (2 cases) or because of evolution of the associated tumour (6 cases). For the 14 subjects with an elapsed time of 2–5 years, 9 were treated for established fibroses, 1 case was interupted, one did not return to the hospital and the remainder (3 cases) still had tumoural evolution and could not be evaluated. With respect to patients with more than 5 years delay (11 cases) all were treated for established fibroses, and in one case two successive treatments with LIPSOD were applied. Thus for the 50 total cases, there were 51 treatments of which 47 allowed judgement of immediate tolerance to LIPSOD and 39 for which efficiency of the treatment could be estimated. This efficacity was studied on fibroses, on related cases such as necroses, edemas of the larynx, and on prefibrotic conditions, that is covering pathological phenomena induced by irradiation other than fibrosis in the strict sense. For some patients, treatment with LIPSOD was initiated when all conditions for the induction of fibrosis were present (high irradiation dose in a large volume with a chronic inflammatory syndrome). In view of the encouraging results, we have recently begun to apply LIPSOD as a preventative measure in the absence of visible clinical symptoms, for example in a case of recuperative curietherapy where the patient had been previously irradiated (at full dose) on several occasions and for whom prognostic of fibrosis as a sequel was certain.

In general the established fibroses resulted from an overdose in a large volume, or

to overlap of different irradiated zones or to underestimation of the entry dose for certain irradiations of the trunk. With certain of the fibroses, treatments anterior to LIPSOD had been attempted particularly by local application of corticoids or of coumarin derivatives (Esberiven). These drugs, especially in association, undoubtedly have an effect on the chronic inflammatory syndrome which precedes formation of the fibrosis but have essentially no effect on an established fibrotic zone.

This series of patients was treated between April 1984 and February 1985. Results were estimated at a functional level and by objective measurement of certain parameters. All the radioinduced anomalies were clinically observable and resulted in a greater or lesser constraint for the patient. Dimensions of the fibroses were easily measurable in 28 cases, with initial limits of 3 to 14.5 cm and an average size of 7.7 cm. When the fibrosis caused movement restriction, improvement was noted as a percentage increase in freedom. Modification of hardness of the fibrosis was estimated on a scale of 0 (no change), + (slight softening) + + (medium softening) and + + + (very pronounced softening). In general, this softening preceded size regression.

Treatment was arbitrarily determined to last three weeks with a twice weekly injection (intramuscular) of 5 mg encapsulated SOD (in two ml) that is, a total of six injections, in order to study possible reversal of an amelioration, continuation of an observed improvement or a static unchanging situation of the reduced fibrosis.

The liposomal superoxide dismutase was prepared from pure non-pyrogenic bovine Cu-SOD (single isozyme) in sterile form. The liposomes are multi-lamellar with an average diameter of 350–400 nm. Preparations are stable at 4°C for at least three years[3].

RESULTS

From a functional viewpoint patients showed a greater or lesser improvement (subjectively expressed) generally proportional to changes in the objective clinical parameters. This improvement began almost always in course of the initial three week treatment.

For 35 fibroses for which dimensions were easily and precisely measurable, the average regression in size was 32%. Evolution of hardness, measurable in 33 cases, showed no change in 6% (2/33), slight softening (+) in 12% (4/33), moderate softening (+ +) in 27% (9/33) and important effects (+ + +) in 55% (18/33) of the fibroses. With respect to dimensions, slight or zero effects were observed in 23% of the cases (8/35) with only two examples in which both hardness and size were unchanged (4%). To summarize, the average size of the fibrosis is reduced by one third and there is a significant softening in 82% of the cases after three weeks treatment with LIPSOD.

With respect to the efficiency of the treatment with LIPSOD as a function of elapsed time since the initial irradiation leading to the fibrosis no difference with respect to the three groups (less than 2 years, 2–5 years, greater than 5 years) could be observed with respect to softening of the fibrosis or reduction of the dimensions. For the two patients with the longest elapsed time, 12 years and 17 years, the former showed a slight softening with no change in size of the fibrose whereas the second (17 years) showed very marked softening and a reduction of the dimensions greater than 40%. Thus the efficiency of LIPSOD on a well established fibrosis is independent of the time between the radiotherapy and the treatment.

It has not proved possible to predict the resistance or not of a given established

fibrosis towards treatment with LIPSOD except that very little success was obtained with 3 cases of "atrophic" fibroses (these are slow to appear, requiring more than five years) whereas plethoric forms with thick fibrous tissue and an edematous bulge around the fibrotic area appear to be particularly susceptible and gave excellent results. Seven patients had two or more fibroses and in three of these cases complete regression was observed for one fibrosis and only a moderate reduction of the other. These differences are perhaps related to the origin of the radiofibrosis since complete regression was seen with those induced by moderate external irradiation only, whereas the more resistant forms were induced by high radiodoses including curietherapy.

In cases with cutaneous atrophy or telangiectasia associated with the fibrosis no changes of these symptoms were observed even when an important regression of the fibrosis occured. For other radioinduced phenomena, two cases of chronic prefibrotic inflammatory syndrome regressed completely, as did the two cases of laryngeal edema. For the two cases of chronic radioepithelitis a functional improvement was observed as well as reduction of the edema and a better limitation of the lesions but not total disappearance, and the LIPSOD appears to act as an anti-inflammatory agent. This was also the case with two subjects with persistent radiomucitis and small necrotic areas for which one was moderately improved and the other markedly ameliorated during the treatment (but later followed by relapse of the necrosis which was then surgically removed).

Preventive treatment with LIPSOD after secondary curietherapy is efficient in that no reactions were observed after the irradiation and 7 months later there are no signs of local infiltration.

After three weeks treatment with LIPSOD and in the present elapsed time since this therapy, it may be noted that no rebound at short or long term has been observed. No reversion occurs and regression of the fibrosis is stable.

Tolerance to treatment with LIPSOD, studied with 47 patients, is in general good, but fever ($\geq$ 38.5°C) has been observed in 15% of the cases (7/47) after the 5th (5 times) or 4th injection, but not after succeeding injections, that is after 2 to 2 1/2 weeks treatment. It may be noted that this relatively high fever rate has not been observed during the treatment of inflammatory diseases in which dissolution of a solid mass is not involved (63 cases treated for various diseases in Japan with no serious side effects). In 6 of the 7 fever reactions the patient showed an important reduction in size of the fibrosis or a very significant softening or a net functional improvement of movement indicating an in depth regression of the fibrosis.

Thus the transitory fever appears to be at least in part associated with regression of the fibrosis in certain cases. That this is not due to production of antibodies against bovine Cu-SOD was readily demonstrated by estimation of such antibodies by radioimmunoassay[4]. Plasma samples from 20 patients including the 7 who had shown a transistory fever greater than 38.5°C were examined[5]. Although antibodies could be detected using this extremely sensitive technique, levels were of the order of 10^{-12} M to 10^{-11} M (4 subjects) or zero (11 subjects), except in five cases where levels ranged from 8×10^{-11} M to 3×10^{-10} M. It may be noted that such levels are insignificant and in comparison with other antibody estimations such as anti-acetylcholine receptor antibodies in cases of myasthenia would be considered as within control values. Nevertheless comparison of the results with appearance of fever was attempted. No correlation existed and among the six highest values only one patient showed fever symptoms. Antibody was not detectable in 4 of the remaining cases of fever. Thus antibody formation does not appear to be a serious problem. Antibody production, at least at

the level of femtomoles per ml, occurs in some of the cases after three weeks treatment, but is not correlated either with fever or therapeutic efficacity. It may be supposed that if antibodies are formed, the superoxide dismutase would be less efficient. Injection of 30 mg encapsulated SOD represents about 10^{-6} moles and given some 5 litres of blood total antibody is 10^4 to 10^6 times less and thus cannot eliminate significant quantities of the enzyme. It may also be supposed that the effective agent is not SOD but rather the complex antibody-antigen (soluble in the case of Cu-SOD) which would presumably have a longer circulation lifetime. This is not the case at least at the levels measurable, in view of the total lack of correlation with efficacity.

No effects of treatment with LIPSOD on the development of cancer, either positive or negative, could be observed with the limited number of cases studies, insufficient to decide whether the relapse-free rate after radiotherapy is modified. If fibrotic tissue encloses and protects a cancerous nucleus it is possible that dissolution could liberate this "dormant" cancer. Again, within the limited number of cases and the time since treatment with LIPSOD this does not appear to be the case.

DISCUSSION

The superoxide radical plays a primary role in initiating and sustaining biological damage and is responsible for the production of other free radicals and lipoperoxides. An initial creation of superoxide (for example by irradiation) can give rise to an autosustaining cascade of activated products with considerable amplification due to free radical chain reactions. While such a situation may rest latent for a long time, perturbation of the system can cause a rapid, almost explosive development, fueled by molecular oxygen, but catalysed by low levels of superoxide radicals.

It may appear surprising that fibroses are "dissolved" by the action of LIPSOD in view of various reports on the depolymerisation of collagen[6] and hyaluronic acid[7] by superoxide radicals *in vitro*. However in such studies the experimental conditions were designed to facilitate observation rather than with relevance to pathological fibrotic conditions. Thus very dilute solutions (10^{-6} M to 10^{-5} M) of the polymers were employed and it is evident that in the case of well established solid fibroses (or indeed of any more or less solid conjunctive tissue) the concentration is much higher. This of course entirely changes the situation with respect to modifications induced by superoxide anions (or secondary radicals such as carbonate anion radicals) in biomacromolecules. In opposition to the depolymerisation studies (which imply that SOD should inhibit dissolution or depolymerisation) may be cited the cross-linking of protein sub-units and of RNA in ribosomes[8], cross-linking of strands in double helical DNA[9] and the polymerisation of hemoglobin, both intramolecular and intermolecular[10] induced by superoxide radicals. The contribution of superoxide to membrane rigidification due to reduced lipid fluidity in microsomal membranes from senescent carnation flowers has also been demonstrated[11].

Thus, in the absence of a convincing explanation of fibrosis formation in the medical literature, we may suppose that initiation and growth of fibrotic tissue results from the action of superoxide radicals (or derived forms of activated oxygen) and that due to the ratio of surface to volume, cross-linking of chondroproteins and other macromolecules to give this solidification should eventually be limited. Because of the extensive three dimensional cross-linking in the solid matrix, lysis by the usual depolymerising enzymes (collagenase, elastase, proteases, etc. . .) is slowed or inhibited and

after several years the final fibrosis may be considered to be in an equilibrium state.

Since we now have a plausible description of the mechanism of fibrotic formation, an explanation of the action of SOD can be elaborated. Once the continuing effect of polymerisation by superoxide is stopped due to fixation of SOD, particularly in the liposomal form, to the outside of the fibrosis, with penetration of about 1 cm in the tissue in the case of LIPSOD (shown by external local application) normal depolymerisation processes can become dominant. It is to be recalled that regression of the fibrosis is often preceded by a decrease in rigidity. In physical terms, depolymerisation to free chains should follow a sigmoid curve. After three weeks treatment regression of certain fibroses is evident and is either total or such that a new reduced equilibrium is reached, stable at least over the time scale presently available. It could well be that at least in some cases, liberation of free chains modified by cross-linkage gives rise to a transitory antigenic situation seen at the fifth injection (i.e. 17 days) but not anterior or posterior, thus giving rise to fever at this time.

Some nuances must nevertheless be introduced in this schema. Fever is seen in few cases only and significant regression is most often free of such secondary effects. Some fibroses are rapidly reduced whereas others are more resistant and both types can exist in the same subject. Whereas regression of the radio-induced fibroses considered in this report gives rise to a stable diminished form after cessation of treatment, this is not the case with pulmonary fibrosis. In such cases short term treatment (i.m. injection of LIPSOD) results in significant amelioration as measured by various clinical parameters such as P_aO_2 but one week to one month after cessation of the treatment dyspnea reappears[12]. A reasonable interpretation is that the role of O_2^- in fibrosis is more or less preponderant and varies from case to case (other mechanisms can of course be envisaged) and that the fine composition of each fibrosis is subject to variation. If a dynamic rather than a static view of an established fibrosis is true then creation of a new stable equilibrium will be subject to local continuing conditions such as neutrophil activity (with production of superoxide), levels of oxygenation or other factors. In this respect, the work of McCord[13] demonstrating transformation of xanthine dehydrogenase (non-producer of O_2^-) to xanthine oxidase (producer of O_2^-) under ischemic conditions due to liberation of a protease is perhaps relevant. Thus respiratory insufficience could be autocatalytic in the formation of fibrotic lung tissue, and reduced vascular circulation in the case of large solid fibroses would not facilitate regression.

From a physiological viewpoint, the toxic actions of high dose radiotherapy on normal tissues have been extensively studied and a number of general rules to reduce damage to a minimum have been established[14,15,16,17]. Late radiolesions appear to arise either from direct cellular damage particularly of highly differentiated cells with slow or zero growth (neurones, hepatic cells), or as a result of vascularitis. This latter is perhaps the major cause of subsequent complications, particularly radiofibrosis of conjunctive tissue. The role of superoxide radicals in vascular damage and increased permeability is well established[18].

The present studies indicate that systemic administration of LIPSOD is particularly active with respect to conjunctive tissue rather than skin or mucous membranes (though preparations designed for local application[2] are very effective). Apart from possible target effects, other factors may play a role. In any case, other pathological processes such as a first phase of chronic inflammation followed by formation of a defined solid phase give rise to fibroses similar to radio-induced fibrosis. It is possible that LIPSOD will prove adequate for the treatment of fibrochondritis, fibromas and

fibromyomas, in which case an increased understanding of the physiopathology of such phenomena may be expected.

The clinical experience with 50 patients (this series is in course of extension to much larger numbers) indicates that LIPSOD has an important anti-fibrotic action on well established radio-induced fibroses in 80% of the cases. Efficacity is most pronounced when the fibrosis is flourishing, in full evolution, and surrounded by an inflammatory edema. At termination of the treatment the situation remains stable. LIPSOD is entirely efficient for the treatment of chronic prefibrotic inflammatory syndromes and inhibits formation of a fibrosis, as does treatment in the latent phase. With respect to prophylactic action in situations where the probability of fibrotic sequels is extremely high the approach appears successful. The action on precocious cutaneous and mucous reactions (chronic persistant radioepithelitis and radiomucitis) appears to be limited to anti-inflammatory effects and no repair is seen (at least by intramuscular injection of LIPSOD). In 15% of the cases a transitory febrile reaction is observed at the 4th or 5th injection, perhaps associated with liberation of degradation products of the fibrosis, since no significant amounts of anti-SOD antibodies could be detected and in cases where extremely small amounts were found, fever was not observed. In six of the seven patients with reactions an important regression of the fibrosis occured. LIPSOD preparations are pyrogen-free in rabbits.

That this fever is **not** associated with simple pyrogenic effects is amply demonstrated by the remarkable efficiency of LIPSOD in **reducing** the duration of fever in babies with Kawasaki disease to one third of the period seen with orthodox aspirin treatment (17 LIPSOD treatments compared with 20 aspirin) using higher dose rates of LIPSOD than generally applied[19].

Apart from treatment of established radio-fibroses, possible prophylactic use in difficult radiotherapeutic situations must be seriously considered, particularly in cases where major organs are involved or unavoidably implicated (e.g. during irradiation for cancer of the oesophagus). Further, therapy of pathological fibroses arising from other causes must also be envisaged if it seems reasonable that superoxide radicals play a role in the etiology. It should perhaps be emphasized that apart from a direct protective action of SOD in the destruction of superoxide ions, indirect effects may also be observed by rectification of a pathological biochemical situation anterior to the final clinical observable result as shown in the treatment of severe hemolytic anemia[1].

Although controls with empty liposomes have not been employed in this study, no effects whatsoever are observed with such material in patients with Behçets disease, Kawasaki disease, severe rheumatoid arthritis, colitis ulcerosa or progressive systemic sclerosis[1] in contrast with the efficacity of LIPSOD. Since no real treatment of long established ratio-induced fibroses has previously been demonstrated, historical comparison is a valid procedure. Nevertheless, it is hoped that placebo studies (using free SOD as the placebo) will be conducted elsewhere.

In this preliminary survey, treatment was limited to 6 injections of 5 mg liposomal SOD over three weeks in order to obtain a maximum of information with a minimum of discomfort for the patients. It is clear that a complete treatment involves application over a longer time period (three months) with less LIPSOD per injection, a protocol which has been successful for other superoxide-related pathologies[1]. Based on animal experiments the optimal quantity per injection is 5–10 μg per kilo[20], that is, about 0.5 to 1.0 mg for an adult human.

The enzymic reaction catalysed by superoxide dismutase is well established and

understood in detail. However, despite the explanations presented above. It cannot be considered that the mechanism of LIPSOD with respect to regression of solid fibroses in humans (or of the more general anti-inflammatory properties) is completely defined. With respect to treatment of patients, the efficacity perhaps outweighs these short-comings, but it is hoped that further work will clarify the fundamental mechanisms involved[21].

Acknowledgements

We thank Dr. L. Flohé and Grunenthal GMBH for a most generous gift of pure bovine copper superoxide dismutase.

References

1. Y. Niwa, K. Somiya, A.M. Michelson and K. Puget, *Free Radical Research Communs.*, in press. (1985).
2. A.M. Michelson and K. Puget, in *Oxygen Radicals in Chemistry and Biology* Eds W. Bors, M. Saran and D. Tait (Walter de Gruyter: Berlin, New-York, 1985) pp. 831-842.
3. A.M. Michelson, in preparation.
4. A. Baret, P. Michel, M.R. Imbert, J.L. Morcellet and A.M. Michelson, *Biochem. Biophys. Research Communs.*, **88**, 337-345, (1979).
5. A. Baret, private communication.
6. R.A. Greenwald and W.W. Moy, *Arthritis Rheum.*, **22**, 251-259, (1979).
7. J.M. McCord, *Science*, **185**, 529-531, (1974).
8. B.S. Cooperman, J. Dondon, J. Finelli, M. Grunberg-Manago and A.M. Michelson, *FEBS Letters*, **76**, 59-63, (1977).
9. C. Monny and A.M. Michelson, *Biochimie*, **64**, 451-453 (1982).
10. J. Thillet and A.M. Michelson, *Free Radical Research Communs*, in press. (1985).
11. S. Mayak, R.L. Legge and J.E. Thompson, *Phytochemistry*, **22**, 1375-1380 (1983).
12. M. Guignier, Centre Hospitaller Regional et Universitaire de Grenoble, private communication.
13. D.A. Parks, G.B. Bulkley, D.N. Granger, S.R. Hamilton and J.M. McCord, *Gastroenterology*, **82**, 9-15, (1982).
14. P. Rubin and G.W. Casarett, *Clinical Radiation Pathology* (Saunders, Philadelphia, 1968).
15. D.C. White, *Cancer*, **37**, 1126-1143 (1976).
16. W.H. Rodney and J.P. Lester, in *Textbook of Radiotherapy* Ed. G.H. Fletcher (Lea and Fibiger, Philadelphia, 1981) pp. 103-219.
17. J.A. Del Regato and H.J. Spjut, in *Cancer* (Mosby, St Louis, 1977) pp. 74-89.
18. R.F. Del Maestro, H.H. Thaw, J. Bjork, M. Planher and K.E. Arfors, *Act. Physiol. Scand.*, **Suppl. 492**, 43-57, (1980).
19. K. Somiya, Y. Niwa, K. Shimoda, S. Fukami, K. Puget and A.M. Michelson, in *Proceedings of SOD IV* Rome September 1985, Ed. G. Rotilio (Elsevier, Amsterdam, 1985) pp.
20. G. Jadot and A.M. Michelson, in preparation.
21. A.M. Michelson, K. Puget and G. Jadot, in preparation.

Accepted by Dr. J.V. Bannister