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ORIGINAL ARTICLE

Nitric oxide and the collagenous protein biosynthesis of irradiated chick chorioallantoic membrane

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Summary

Purpose: *To study the effect of irradiation doses (2 Gy and 5 Gy) on the rate of collagenous protein biosynthesis (CPB) of chick embryo chorioallantoic membrane (CAM) and to investigate the possible role of the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), its inactive enantiomer D-NAME and the NO donor sodium nitroprusside (SNP) in this effect.*

Materials and methods: *The originally established CAM angiogenesis model was used with few modifications described earlier. The CAM areas were irradiated on the 10th or the 14th day of embryo development with a single dose of 2 or 5 Gy and the CPB was determined 6 h later. L-NAME, D-NAME and SNP were added to the CAM after the irradiation.*

Results: *The experiments didn't show any significant differences in almost all of the treatment courses. In the 10-day CAM preparations the irradiation not significantly (NS) inhibited the CAM CPB, whereas L-NAME abolished this effect only in the case of 2 Gy dose (NS). The addition of SNP or D-NAME in the post-irradiated 10-day CAM exerted radiosensitization that was significant only in the case of the combination of D-NAME with the 5 Gy dose. There was no significant effect on the respective treatments of the 14-day CAM. The CPB of the 14-day CAM was significantly lower with regard to the values of the 10-day CAM.*

Conclusion: *The biochemical evaluation of the CAM (CPB method) seems to be not suitable for radiobiological studies. Nevertheless, the implication of NO in the X-ray induced antiangiogenicity cannot be excluded.*

Key words: angiogenesis, chorioallantoic membrane, collagenous protein biosynthesis, irradiation, nitric oxide

Introduction

One of the most widely applied systems for studying angiogenesis *in vivo* is the chick embryo CAM [1,2]. The preference for this system in assessing morphological and functional changes in vessels under normal or experimental conditions is due to its advantages, such as simplicity, reproducibility and usefulness [3,4]. The angiogenesis in the CAM model can be also determined via the monitoring of the CPB [5,6]. It is known that collagenous proteins represent 80% of the total basement membrane proteins formed by the CAM [5]. The extent of their biosynthesis has been shown to correlate with the formation of new blood vessels, so the biochemical method for the measurement of the CAM's CPB is regarded to be an indirect index of angiogenesis [6]. Although both the morphological and biochemical method for studying angiogenesis in the CAM are useful, it seems that the second method has not been adequately used in radiobiological experiments with the CAM [3,7,8].

NO is chemically a free radical that has a diverse range of actions in both physiological and pathological processes [9,10]. NO is also suggested to be involved in the regulation of CAM angiogenesis [11,12]. Previous results have shown, using the single dose of 10 Gy, that, in the CAM model, X-rays mediate their antiangiogenic effects through an action on NO synthase [3]. In addition, 2 other studies that described the irradiation dose-response (X-rays ranged from 2 to 15 Gy)

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effect on the CAM confirmed the implication of NO in the mode of action of irradiation [4,13].

The current study sought to investigate the effect of 2 irradiation doses (2 Gy and 5 Gy) on the rate of CPB of the CAM and to examine the possible role of the NO synthase inhibitor L-NAME, its inactive enantiomer D-NAME, and the NO donor SNP in this effect.

Materials and methods

Materials

Fresh fertilised eggs were obtained locally (Ioannina, Greece). L-NAME, the inactive enantiomer D-NAME, SNP, collagenase type VII from *clostridium histolyticum* and cortisone acetate were purchased from Sigma Chemical Co (Poole, UK). The L-[U-^{14}C] proline was purchased from Amersham (UK). All other chemicals used were from Merck (Germany) or BDH (Poole, UK).

Angiogenesis in vivo

Folkman's CAM angiogenesis model [2,14] was further modified from a previous work [3]. The ring modification was not used in the current series of experiments [4]. Briefly, fresh fertilised eggs were externally sterilised with 70% isopropanol and placed at a slanted position in the egg case. This position was maintained during the following incubations. The incubation at 37° C and 55% humidity was carried out for 4 days, at the completion of which a window (1×2 cm) was opened on the egg shell, exposing the CAM. The window was covered with adhesive tape and the incubation continued up to the 10th or the 14th day of embryo development, when a part (1.7 cm^2) of the CAM was irradiated with a single X-ray dose of 2 or 5 Gy by means of a Philips irradiator (20 kV, 0.1 mm Al) to avoid harming the embryo. Approximately 20 min after the irradiation one sterilised plastic disc (diameter 1 cm, from an overhead projector film, Pelikan #120) was used to cover the irradiated CAM area and a second disc to cover an adjacent (non-irradiated) CAM area in order to serve as control. All discs contained sterile cortisone acetate (100 μg) to prevent local inflammation [15], in addition to the test compound (L-NAME, D-NAME or SNP). L-NAME, D-NAME and SNP were used according to previous studies in the doses of 3.3, 3.3 and 5.0 μg per disc, respectively [3,4,13]. Six hours post-irradiation the area under each disc was cut off, immersed in buffer and the protein biosynthesis interrupted. In our experiments, we studied the effects of ionizing radiation on chick embryo CAM from day 10 (actively proliferating CAM) and day 14 (quiescent CAM). Based on data from previous experiments, the 6th hour after irradiation was chosen as the optimal time point for the CPB evaluation of the CAMS [3]. After washing off non-protein radioactivity, the pellets containing the protein radioactivity were digested with collagenase. The resulting tripeptides from the basement membrane and other CAM collagen were counted by liquid scintillation analyzer (Wallac 1410 radioactivity counter) and expressed as dpm mg^{-1} CAM site protein (dpm stands for disintegrations per minute).

Statistical methods

When paired observations (from the same egg) were used, we employed the intra-assay or paired Student's t-test, whereas, when CAM data were compared between different eggs, the unpaired Student's t-test was used [16,17]. A Microsoft Windows program (Microcal origin version 2.94, Microsoft Corporation) was used for the calculations by means of a computer.

Results

Figure 1 shows the difference in the rate of CAM CPB on day 10 and 14. The mean value for the CPB of the 10-day CAM was 15725.6 ± 2144.2 dpm/mg protein, whereas the respective one for the 14-day CAM was 2878.1 ± 963.1 dpm/mg protein. The difference between them was significant ($p < 0.001$). Angiogenesis, assessed by morphological and biochemical methods in

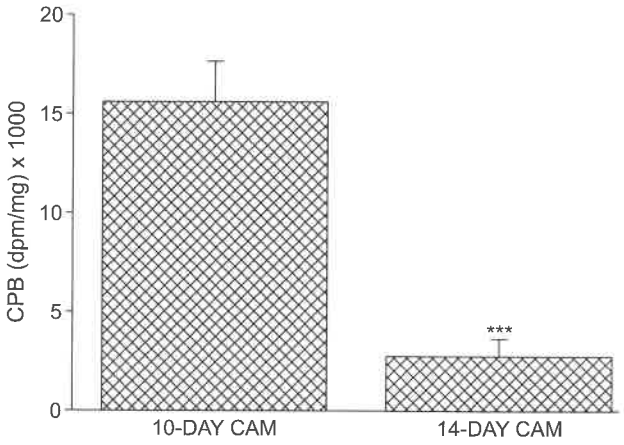


Figure 1. Comparative barograph showing the significant difference of the collagenous protein biosynthesis (CPB) rate between non-irradiated 10-day and 14-day CAM. Results are expressed as mean ± S.E. for CPB. The asterisks (***) indicate paired t-test significance ($p < 0.001$).

the CAM model, reached a maximum between days 9 and 12 and after day 14 showed a plateau. Table 1 summarizes the results obtained by the various combinations of the current experimental design.

The NO-synthase inhibitor L-NAME, the inactive enantiomer D-NAME, and the NO-donor SNP were applied in the above mentioned concentrations and in combination with X-irradiation to the 10- and 14-day CAM (Table 1). Single doses of 2 or 5 Gy in combination with the above agents did not significantly affect the CPB of almost all the preparations of the 10- and 14-day CAM. Single irradiation produced dose -dependent but not significant antiangiogenesis (-9.3 and -24.3% for 2 and 5 Gy, respectively) in the 10-day CAM. Although L-NAME in combination with irradiation did not produce any significant effect in the 10-day CAM, the values of CPB in these experiments appeared not to be affected by irradiation, i.e. L-NAME seemed to have a non-significant radioprotective action to the 10-day CAM CPB. D-NAME as well as SNP did not exert such an action. Both of them failed to change significantly the CPB of the irradiated 10-day CAM but, generally, a slight radiosensitization was observed. In the case of the combination of D-NAME with the dose of 5 Gy this radiosensitisation (-34.9% on CPB value) was significant ($p < 0.05$).

The experiments regarding the 14-day CAM gave results dissimilar compared to the 10-day CAM preparations results. Single irradiation produced dose-dependent but not significant increase of CPB values (+15.9 and +32.2% for 2 and 5 Gy, respectively), whereas L-NAME and D-NAME exerted a relative radiosensitisation on the CPB of the 14-day CAM. On the other hand, SNP showed to provoke a non significant increase of the 14-day CPB values. Nevertheless, as mentioned above, the CPB values of the 14-day CAM were extremely lower with regard to the values of the 10-day CAM. In addition, the profile of CPB in the 14-day CAM may evoke different mechanisms of reaction to irradiation.

Discussion

Angiogenesis is now appreciated as an "organizing principle" in biology with important role in various diseases [18]. The elucidation of the angiogenetic processes will probably give insight into future drug development. In parallel, this may lead to optimization of the cancer pharmaceutical therapy and radiotherapy. One of the main models for studying angiogenesis is the chicken CAM. The scope of the current study was to investigate the CPB measurement approach to radiobiological experiments using the CAM. The effect of L-NAME, D-NAME and SNP on CAM irradiation with doses of 0-5 Gy was also studied, in order to follow up earlier results indicating that at 10 Gy the irradiation-induced reduction of CAM vascular density and CPB is NO-dependent [3].

Table 1. Effect of irradiation dose-response studies (2 or 5 Gy), in the presence or absence of L-NAME, D-NAME or SNP, on the CAM collagenous protein biosynthesis (CPB) on day 10 or 14. Results are expressed as mean ± S.E. for CPB, as well as the difference percentages between treatment and the control areas of the respective CAM's. The symbol NS indicates non significant paired t-test difference compared to the non-irradiated controls (n= number of eggs)

CAM Day	*Combination*	*CPB Control ± SE (dpm/mg)*	*CPB Treatment ± SE (dpm/mg)*	*CBP ± SE (%)*	*n / Significance*
10th Day CAM	2Gy	11925.3±3648.6	10808.1±1543.4	–9.3±12.8	n=6-7 / NS
	2Gy + L-NAME	9252.4±2225.7	13704.6±2586.8	+48.2±28.0	n=6-7 / NS
	2Gy + D-NAME	15230.7±2964.2	9675.9±755.0	-36.5±5.0	n=6-7 / NS
	2Gy + SNP	28401.7±6063.9	17565.5±2420.3	-38.1±8.5	n=6-7 / NS
	5Gy	20352.7±5969.7	15408.1±3129.4	-24.3±15.3	n=6-7 / NS
	5Gy + L-NAME	16312.3±3798.7	16577.9±3832.2	-1.6±23.5	n=6-7 / NS
	5Gy + D-NAME	23116.5±2698.5	15049.7±2040.1	-34.9±8.8	n=6-7 / NS
	5Gy + SNP	15548.0±4170.3	10805.1±3507.9	-30.5±22.5	n=6-7 / p<0.05
14th Day CAM	2Gy	1566.8±404.2	1806.8±336.3	+15.9±21.2	n=6 / NS
	2Gy + L-NAME	1307.8±314.8	1164.6±454.4	-11.2±34.4	n=6 / NS
	2Gy + D-NAME	2649.3±721.7	1233.6±243.0	-53.8±9.2	n=6 / NS
	2Gy + SNP	1745.3±322.0	1758.8±256.0	+0.9±14.6	n=6 / NS
	5Gy	1314.8±465.1	1741.8±656.2	+32.2±50.2	n=5-7 / NS
	5Gy + L-NAME	1795.8±520.6	844.2±202.2	-53.0±11.2	n=5-7 / NS
	5Gy + D-NAME	6569.7±2948.9	2473.3±722.9	-62.3±11.0	n=5-7 / NS
	5Gy + SNP	1201.5±304.7	1445.0±415.3	+20.0±33.7	n=5-7 / NS

For abbreviations see text

The difference between the rate of CAM CPB on day 10 and 14 was significant ($p < 0.001$) and confirms previous results [3,5,6].

The combination of X-ray irradiation and the L-NAME applied post-irradiation inhibited (not significantly) the irradiation-dependent reduction of CPB in the case of the 10-day CAM. The reduction of the 10-day CAM CPB was irradiation dose-dependent. An additive antiangiogenic effect with the irradiation doses of 10 and 15 Gy has not been shown in previous studies, probably because above 5 Gy all the irradiation-sensitive small vessels are already destroyed [3,4]. The pattern in the 14-day CAM was not similar with that of the 10-day CAM. The 9-day and 10-day CAM show the optimum rate of CAM angiogenesis, whereas the 14-day CAM is angiogenetically quiescent [3]. Irradiation had no significant effect on the 14-day CAM as shown in our earlier single dose studies, because irradiation affects mainly the proliferating cells [4]. The present results on 14-day CAM had no consistency and showed that the CPB method may not be easily useful in radiobiological experiments. Possibly, the regional vasoconstricting effect of L-NAME, due to hypoxia, provides a radioprotective effect to the irradiated 10-day CAM area, in agreement with previous studies [3,4,13]. It can be hypothesized that elucidation of the NO-dependent mechanisms in tumors and surrounding normal tissue may improve radiotherapy if X-ray irradiation is appropriately combined with chemotherapy for NO modulation [19,20]. Apart from the important implications of NO in the irradiation and angiogenesis mechanisms, the vasodilatory effect of NO during tumor radiotherapy may reduce tumor hypoxia and act as a radiosensitizer [19,20]. However, extensive and thorough investigation of NO diverse implications in live systems is still needed [21]. The possible hydroxyl radicals scavenging properties by inhibitors of NO synthases might be also an important mechanism of radioprotection [22]. Noteworthy, a recent study identifies NO as a facilitator of collagen synthesis and promoter of angiogenesis in burn wounds [23].

The ring method (an additional modification of the typical CAM disc methodology) described for the first time by us [4], offers the opportunity to explore further parameters implicated in the rate of CAM's CPB. Accordingly, the use of other free radicals scavengers or inducers will probably prove convenient for the study of NO involvement during CAM irradiation. The example of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a paramagnetic nitroxyl spin probe and free radical scavenger which was initially used by our team on CAM studies, may encourage the experimental investigation towards that pathway [24]. Recently, TEMPOL and other antioxidants have been used in experiments in order to elucidate the role of reactive oxygen species in the CAM vascular damaging effects of X-rays [22]. Interesting are the results of the recent completion of a phase I study which investigated the effect of NO synthesis on tumor blood volume and vascular activity [25]. The study provided clinical evidence that inhibition of NO synthesis has tumor antivascular activity [25].

In conclusion, the current study showed that the biochemical evaluation (CPB method) of the CAM may not be suitable for radiobiological experiments. Furthermore, the role of NO in the antiangiogenic effects of X-rays is highlighted as an important target for radiopharmacological research.

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