RNA Metabolism, DNA Damage and Cellular Resistance to X-Rays: Investigations in Chick Embryo and Rat Cells

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Three hours after X-irradiation *in vivo* with 8 Gy the *in vitro* incorporation of [^{3}H]uridine into total RNA of liver(L)- and brain(B)-cells of the chick embryo was reduced to 77% and 90%, respectively; the mRNA fraction was strongest inhibited. Under the same conditions, protein synthesis of L-cells declined to 62%, while protein synthesis of B-cells was not influenced. RNA and protein metabolism was not altered following X-irradiation *in vitro* (1.75–56 Gy). – Compared to thymic- and splenic cells of the rat, chicken embryo cells exhibited higher constitutive poly(adenosine diphosphate-ribose)polymerase activity and lower X-irradiation-induced DNA damage. – Whereas the slight inhibition of RNA and protein synthesis by X-irradiation in ovo may be an abscopal and/or secondary phenomenon reflecting DNA and/or cellular damage, the present investigations comprising various cell types argue for an efficient DNA repair in chicken embryo cells caused, at least partly, by a high constitutive activity of DNA repair proteins.

Introduction

Among vertebrates, avian species are more resistant to ionizing radiation than mammals [1]. This seems also to be true for avian embryos [2–4]. Though tissues of high mitotic activity – like the proliferating cell populations in the embryo – are more responsive to irradiation than non-dividing differentiated cells [5], the mean $LD_{50/24h}$ of the chicken embryo throughout the 21 days of in ovo development is about 8 Gy [2–4] as compared to acute LD_{50} values of 1–2 Gy for the mammalian embryo [6].

The reasons for the relatively high radiation resistance of the avian embryo remain to be investigated. Previous studies of our laboratory in chick embryo liver and brain on the 12th to 15th day of development [7, 8] suggested an efficient DNA repair system, but rapid cell replacement repair following necrotic and/or apoptotic phenomena couldn't be excluded [8].

There exists some evidence that changes in RNA and protein metabolism may play an important role in radiation induced repair phenomena. Investigations in thymocytes, for instance, suggested that breakdown of newly formed RNA may be responsible for interphase (apoptotic) cell death [9, 10]. Chiu *et al.* [11] concluded "that transcriptionally active DNA sequences are more susceptible to ionizing radiation-induced damage than are inactive sequences". In their review, Terleth *et al.* [12] postulated "preferential repair of transcriptionally active DNA". The results of Munson and Woloschak [13] demonstrated an abnormal regulation of transcription in gut tissues of repair deficient mice after ionizing radiation.

Our principal approach has been 1) to assess the influence of X-irradiation *in vitro* and *in vivo* on RNA and protein metabolism of chick embryo liver and brain cells 2) to reveal, in comparison with mammalian cells of comparably high mitotic activity, a possible correlation between transcriptional activity of the chicken embryo cells and X-irradiation-induced DNA damage, which has been measured by nucleoid sedimentation and DNA synthesis. Additionally, the constitutive activity of an enzyme playing a fundamental role in a number of nuclear functions such as DNA repair, differentiation and gene expression, poly(adenosine diphosphate-ribose)polymerase, was determined.

Materials and Methods

Chemicals

[5,6-^{3}H]uridine ([^{3}H]U, specific activity 1.48 TBq/mmol), L-[4,5-^{3}H]leucine ([^{3}H]L, specific activity 1.92 TBq/mmol), and nicotinamide-

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[U-^{14}C]adenine dinucleotide ([^{14}C]-NAD, ammonium salt, specific activity 11.0 GBq/mmol) were purchased from Amersham Buchler (Braunschweig, F.R.G.). The other substances were bought from Merck (Darmstadt, F.R.G.), Serva (Heidelberg, F.R.G.), Sigma (München, F.R.G.) and EGA-Chemie (Steinheim, F.R.G.).

Animals, cell preparation

We used fertile eggs from White Leghorn chickens and female Wistar rats (180–200 g body weight). The embryos were removed from the eggs and sacrificed by decapitation at the stage of 15 days after beginning of incubation. For every experimental group four fertile eggs were used.

Liver cells were prepared as described earlier [8]. *Brain cell* suspensions were made by the same procedure without collagenase. The cell suspensions were adjusted to final concentrations of $1 \times 10^6 - 12.5 \times 10^6$ cells/ml providing comparable RNA- and DNA contents. Cell viability was determined by trypan blue dye exclusion. Furthermore, as mammalian cells, rat thymic (T) and splenic (S) cells were prepared as previously described [14].

X-irradiation

For *X-irradiation in vivo,* the Kristalloflex 710 H X-ray machine (Siemens, Erlangen, F.R.G.) was applied (55 kV, 28.2 mA, half value layer 0.16 mm Cu, filtration with 0.16 mm Cu) at a surface dose rate of 0.4 Gy/min. For dosimetry we used a small ionization chamber (Siemens Egdiv 891a, F.R.G.). Control measurements in a hard boiled egg showed 67% of the surface dose in the yolk. Embryos were exposed either to a single surface X-ray dose (0.25, 1, 4, 8 or 10 Gy) or to a fractionated X-ray exposure (4 or 8 Gy, given as 0.5 or 2 Gy per day at 4 consecutive days) and were sacrificed at various times after irradiation. The control eggs were sham-irradiated. For *X-irradiation in vitro,* 1 ml aliquots of the cell suspensions were X-irradiated in micro test tubes (Eppendorf Safe Lock 0030 120094) placed on ice at a dose rate of 1.75 Gy/min (1.75–56 Gy). Control preparations were sham-irradiated.

Analytical procedures

RNA synthesis (RNS) was determined by the incorporation of [^{3}H]U in the acid insoluble, hydrolysable cell fraction. To 0.5 ml of the cell suspensions [^{3}H]U was added (50 µl with an activity of 37 kBq), and the cells were incubated for 45 min at 37 °C. For extraction of RNA the method of Castles *et al.* [15] was slightly modified: The incorporation of [^{3}H]U was stopped by the addition of cold perchloric acid (PCA) to a final concentration of 0.125 mol/l. After centrifugation (5 min at $9{,}000 \times g$, 4 °C) and washing (three times in 0.125 mol/l PCA, 4 °C), the precipitate was dissolved in 1.5 ml NaOH (1 mol/l) at room temperature. After 1 h 0.5 ml HCl (4 mol/l) were added, and than the samples were centrifuged (10 min at $900 \times g$). The supernatant (hydrolyzed [^{3}H]U-RNA) was safed to quantify the [^{3}H]U incorporation by liquid scintillation counting in Ready-Solv EP (Beckmann 158729). RNA in this supernatant was read at 260 nm against hydrolyzed yeast RNA standards.

The *degradation of RNA,* which had been marked previously with tritiated uridine, was determined by the time-dependent diminution of the activity in the precipitable cell fraction [9, 10].

For *RNA preparation* total RNA was isolated from liver cells which previously had been marked with [^{3}H]U during an incubation period of 90 min at 37 °C (20×10^6 cells/ml, 370 kBq [^{3}H]U) by the LiCl-urea technique [16, 17]. RNA was partitioned in 2 fractions representing the mRNA and rRNA/tRNA fractions by chromatography using oligo-dT cellulose (Boehringer) (18). Aliquots of each RNA fraction were collected to quantify RNA concentration by absorption at 260 nm and [^{3}H]U incorporation by liquid scintillation counting.

The incorporation of [^{3}H]leucine ([^{3}H]L) into perchloric acid precipitates was used as a measure of over-all *protein synthesis* [19]. The protein concentration was determined by the Bradford method (Bio-Rad kit, Munich, F.R.G.).

Protein degradation was determined by principally the same method as for RNA degradation. After marking the protein with [^{3}H]L the time-dependent diminution of the activity in the precipitable cell fraction was determined.

Poly(adenosine diphosphate-ribose)polymerase (PADPR) activity was assessed in permeabilized cells by the incorporation into the acid-insoluble precipitates of the adenosine diphosphate (ADP)-ribose portion of [^{14}C]-NAD [20]. For *nucleoid sedimentation (SED)* the technique was performed as described for T- and S-cells of the rat [21].

Results

X-irradiation in vitro (1.75–56 Gy) had only little effect on RNA synthesis. Even at the highest doses used the tritium activity of the RNA of L-, T-, and S-cells was not significantly altered 0–120 min following exposure; in B-cells the [^{3}H]U incorporation was slightly reduced to 83% of the control values (Table I).

In further experiments the [^{3}H]U incorporation in chick embryo cells was studied 0, 3, 6, 12, and 24 h after a single dose of 8 Gy *in vivo*. The incorporation of [^{3}H]U in RNA of L-cells was strongest inhibited 3 h after a single dose of 8 Gy (to 77% in L-cells and to 90% in B-cells) (Table II). 24 h after exposure no significant inhibition of RNA synthesis was found. The specific activities of total RNA, rRNA/tRNA and mRNA of L-cells 3 h following X-irradiation in ovo were reduced to 61, 69 and 44% of untreated controls (Table III). The greater inhibition of tracer incorporation into total RNA revealed by the LiCl-urea technique might be caused by the different experimental conditions.

The dose dependence of radiation-induced effects on RNA synthesis was examined 3 h after exposure (Table IV): In L- as well as in B-cells significant effects were found only after single doses greater than 4 Gy (78 ± 4% in liver cells, 80 ± 7% in brain cells).

Fractionated X-irradiation on four consecutive days (0.5 and 2 Gy, as a whole 2 and 8 Gy resp.) had no influence on the incorporation rate of tritium 3 and 24 h after the last fraction.

Table I. Mean (± standard deviation) [^{3}H]U incorporation (percentage) into RNA of chick embryo L- and B-cells and rat T- and S-cells immediately following *in vitro* irradiation. The values represent the mean of two to four different experiments which were done in triplicate. Untreated controls = 100%.

X-ray dose [Gy]	Percentage [^{3}H]U incorporation after a dose *in vitro*			
	L-cells	B-cells	T-cells	S-cells
1.75	98 ± 1	96 ± 3	n.d.	n.d.
3.5	96 ± 4	98 ± 3	n.d.	n.d.
7.0	100 ± 3	94 ± 5	91 ± 9	106 ± 3
14.0	93 ± 5	95 ± 3	102 ± 5	96 ± 5
28.0	94 ± 6	83 ± 5	95 ± 6	98 ± 1
56.0	108 ± 8	83 ± 7	94 ± 3	98 ± 3

n.d. = not determined.

To examine the effect of X-irradiation on RNA breakdown, the loss of PCA precipitable activity was measured at various time intervals

Table II. Mean (± standard deviation) [^{3}H]U incorporation (percentage) into RNA of chick embryo L- and B-cells determined in the acid-insoluble hydrolyzable cell fraction [15] following *in vivo* irradiation as a function of time. The values represent the mean of two to four different experiments which were done in triplicate. Untreated controls = 100%. t-test: n.s. = no significance.

Time after irradiation [h]	Percentage [^{3}H]U incorporation after a dose of 8 Gy	
	L-cells	B-cells
0	85 ± 4 ($p < 0.05$)	92 ± 2 ($p < 0.01$)
3	77 ± 6 ($p < 0.01$)	90 ± 6 ($p < 0.05$)
6	86 ± 5 ($p < 0.01$)	88 ± 9 ($p < 0.05$)
12	87 ± 8 ($p < 0.05$)	81 ± 14 (n.s.)
24	99 ± 9 (n.s.)	91 ± 19 (n.s.)

Table III. Mean (± standard deviation) [^{3}H]U incorporation in mRNA and rRNA/tRNA fractions of L-cells revealed by the LiCl-urea technique [17] 3 h after X-irradiation (8 Gy) *in vivo*. The values represent the mean of two experiments. The values in parenthesis stand for per cent of the untreated controls.

	[^{3}H]U incorporation (dpm/mg RNA)	
	rRNA/tRNA	mRNA
Controls	758,363 ±208,196 (100 ± 27)	6,592,857 ±3,040,145 (100 ± 46)
X-Irradiation	526,288 ±203,450 (69 ± 27)	2,897,803 ±1,946,365 (44 ± 29)

Table IV. Mean (± standard deviation) [^{3}H]U incorporation (percentage) into RNA of chick embryo L- and B-cells determined in the acid-insoluble, hydrolyzable cell fraction [15] following *in vivo* irradiation as a function of dose. The values represent the mean of two to four experiments which were done in triplicate. Untreated controls = 100%. t-test: n.s. = no significance.

X-Ray dose [Gy]	Percentage [^{3}H]U incorporation 3 h after a dose *in vivo*	
	L-cells	B-cells
1.00	98 ± 5 (n.s.)	98 ± 8 (n.s.)
2.00	94 ± 5 ($p < 0.05$)	107 ± 3 (n.s.)
4.00	78 ± 4 ($p < 0.01$)	80 ± 7 ($p < 0.01$)
8.00	77 ± 6 ($p < 0.01$)	90 ± 6 ($p < 0.05$)
10.00	76 ± 5 ($p < 0.01$)	81 ± 6 ($p < 0.01$)

(0–120 min) after labeling the cells with [^{3}H]U. RNA degradation after X-irradiation *in vitro* (8 Gy) and *in vivo* (3 h after 8 Gy) was the same as in untreated controls (results not shown).

Within 3 h after a single dose of 8 Gy *in vivo* [^{3}H]L incorporation in the protein fraction of L-cells declined to 62% of untreated controls, while the protein synthesis of the B-cells was not influenced (101%). The protein catabolism was investigated after a single dose of 8 Gy *in vitro* by the loss of PCA precipitable radioactivity at various intervals after labeling the cells with [^{3}H]L. No significant differences existed between the X-irradiated embryos and the sham-exposed controls.

Whereas the overall transcription activity may be without relevance in defining the cellular radiation sensitivity, evidence is increasing that the synthesis of specific proteins occupying a key position in nucleic acid synthesis and repair has to be considered as an important determinant (see, *e.g.*, [22]). Therefore, the constitutive activity of an enzyme system known to play a fundamental role in various nuclear functions including DNA repair, poly(adenosine diphosphate-ribose)polymerase (PADPR) [23], was determined in chick embryo and rat cells, resp. DNA damage was measured by nucleoid sedimentation. As presented by Table V, the avian cells showed a substantially higher [^{3}H]U incorporation than the mammalian cells. PADPR-activity exhibited values which were three- to five-fold higher in L- and B-cells as compared to T- and S-cell preparations. With respect to nucleoid sedimentation, X-ray doses of 6 Gy decreased the sedimentation distance of L- and B-cell nucleoids by 25%. About five to ten-fold lower doses were sufficient to produce the same effect in T- and S-cells.

Table V. Mean (± standard deviation) [^{3}H]U and [^{14}C]NAD utilization for RNS and PADPR, respectively, and DNA damage measured by nucleoid sedimentation (SED) in L-, B-, T-, and S-cells. In general, the values represent the mean of 10 to 15 experiments *(n)* which were done in triplicate.

Assay	L-cells	B-cells	T-cells	S-cells
RNS	219.9 ±108.9	247.5 ±116.6	124.4 ±9.5	101.4* ±8.3
PADPR	181.4 ±59.1	144.3 ±58.6	37.1 ±9.9	53.3* ($n = 1$)
SED	6.0 ±1.5	6.0 ±1.4	1.25 ±0.4	0.6** ±0.2

* The values are expressed as pmol of the incorporated tracer per mg DNA.

** The values are expressed as DE_{25} in Gy (doses with 25 per cent effectivity as related to the sham-irradiated control cells).

Discussion

Radiation effects on RNA synthesis vary widely with tissue type, animal species, RNA species, radiation quality, dose, and postirradiation time [24]. Compared to the large number of investigations in laboratory animals, *e.g.*, rat and mouse, only a few studies have been done in the chick embryo with results varying from increased [25], unchanged [26] or decreased [27] RNA synthesis after irradiation.

In a first series of experiments, the influence of X-irradiation *in vitro* and *in vivo* on RNA and protein synthesis in liver and brain cells of the chick embryo was investigated. The results show, that *X-irradiation in vitro* had little influence on [^{3}H]U incorporation (Table I) which seems to exclude an immediate effect on the transcription process. *In vivo,* however, total RNA synthesis was inhibited by about 20%, the effects being greatest 3–6 h after exposure to single doses of 4–8 Gy and in purified mRNA (Tables II–IV); fractionated X-irradiation had no influence on RNA synthesis. Compared to the investigations of Stammberger *et al.* [8], who demonstrated that 3 h following X-irradiation of chick embryos with doses of 4 and 8 Gy scheduled DNA synthesis of hepatocytes was reduced to about one-third, total RNA synthesis appeared less radiosensitive than DNA synthesis. This has also been observed by other authors (see, for example, [28]). It is probable therefore that the slight inhibition of RNA synthesis 3 h following X-irradiation *in vivo* may be an abscopal and/or secondary phenomenon reflecting cellular damage rather than a specific effect on RNA transcription.

Autoradiographic and histological studies on livers of X-irradiated chicken embryos argue for reparative proliferation adjacent to cell necrosis, though apoptotic phenomena could not be excluded [8]. Programmed cell death (apoptosis) occurs spontaneously in embryonic and adult tissues [29–31] and can be triggered by certain pathological stimuli like X-irradiation [32–34]. The mechanism of apoptosis is still obscure. Apart from this, there

is some evidence, that RNA and protein metabolism could play a significant role in naturally and induced programmed cell death [9, 10, 29]. Geraci and co-workers [9], for instance, suggested that the observed radiation-induced catabolism of RNA in thymocytes of rats may be responsible for interphase cell death. In the present study, however, acceleration of RNA- and protein degradation could be observed after X-irradiation neither *in vitro* nor *in vivo*. Therefore, in the chick embryo, no biochemical evidence exists for programmed cell death induced by increased RNA depolymerisation.

As recently evidenced in UV-irradiated eukaryotic and prokaryotic cells, actively transcribed genes are several times more rapidly repaired than nontranscribed genes [12, 35, 36]. Hanawalt suggests that "the viability of mammalian cells may be ensured through selective repair of transcription-blocking DNA damage in essential expressed genes rather than as a consequence of overall genomic repair" [36]. Whereas in the present investigations the total RNA-synthesis, though being significantly higher in L- and B-cells as compared to the rat cells, don't reveal substantial cell-specific pecularities (Table V), the possibility should be considered therefore that in chicken embryo cells specific genes regulating the synthesis of DNA repair proteins are expressed to a significantly higher extent than in T- and S-cells, and that these transcriptional activities determine – at least partly – the higher radiation resistance of the chicken embryo. This suggestion is supported by the following reasons: 1) As presented by Table V, chick embryo cells, when compared to T- and S-cells, display an about three- to four-fold higher PADPR activity. The same is true as to the hydroxyurea-resistance of L- and B-cells [8] reflecting probably a high constitutive activity of a further key enzyme in DNA synthesis, ribonucleotide reductase [37]. 2) When chicken embryo cells are X-irradiated *in vitro,* the nucleoid sedimentation assay indicates threshold doses which are at least four-fold higher than the equieffective doses in T- and S-cells (Table V). 3) As compared to T- and S-cells, chicken embryo cells exhibit an at least four-fold increased radiation response of DNA synthesis (SDS) [38]. From a number of investigations as reviewed, *e.g.*, by Lavin and Schroeder [39], it has been concluded that damage resistant DNA synthesis – like SDS in T- and S-cells – is able to sensitize many eukaryotic cell species to ionizing radiation or radiomimetic agents, an extensively studied example being cells of Ataxia Telangiectasia-patients.

The comparison of chicken embryo liver and brain cells with T- and S-cells of the rat may be considered as not relevant for species comparison as lymphatic cells are generally more radiosensitive than other somatic cells. The criterion of comparison, however, was mitotic activity, being a fundamental determinant in radiation sensitivity [5]. Apart from this, bone marrow cells of the rat, with respect to SDS and nucleoid sedimentation, responded to X-irradiation *in vitro* closely similar to T- and B-cells [40].

Taken together, the results argue for a DNA repair mechanism, confering in DNA damage a "quasi-threshold" [41] at doses of 2–4 Gy in chicken embryo cells. Whereas the present paper demonstrates that the overall transcription inhibition of chick embryo cells following X-irradiation in ovo may be a rather abscopal and/or secondary phenomenon, the results suggest that the percentage of specific transcriptional activities have to be considered as a substantial determinant in the radiosensitivity of eukaryotic cells. In this context, DNA repair phenomena should be investigated in genes which are selectively transcribed in different eukaryotic species, tissues and developmental stages of definite radiation sensitivity. Additionally, investigations should comprise the determination of specific, possibly inducible DNA repair enzymes.

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