

Malformations after radiation exposure of preimplantation stages

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ABSTRACT Our studies have shown that, contrary to the opinion in most textbooks, it is possible to increase the number of malformed fetuses in one of our mouse strains (originally "Heiligenberger Stamm", meanwhile HLG/Zte) by radiation exposure of zygotes or of subsequent preimplantation stages. The malformation affected most pronouncedly is gastroschisis, a defect occurring at a frequency of 1 to 4% in the controls. The observed increase is strain specific (C57Bl mice or (HLGxC57Bl)_F₁ hybrids do not react in the same way), it is accompanied by an increased frequency of chromosomal aberrations in skin fibroblasts and of modified protein patterns in liver, kidney, and skin cells of day 19 fetuses. The most probable explanation seems to be the assumption that radiation exposure of preimplantation stages increases a defect with a genetic predisposition in a specific way and labilizes the genome of subsequent cell generations making these cells more susceptible for noxes acting on the fetus.

KEY WORDS: *malformation, preimplantation stage, genetic instability, genetic predisposition, radiation risk*

Introduction

The high radiosensitivity of developmental processes has been demonstrated in many studies and radiation induced changes are important for the identification of fundamental developmental mechanisms as well as for the evaluation of radiation risk.

In 1935, Job concluded from his experiments, in which preimplantation stages of rats were exposed to ionizing radiation, that these early embryos either died or survived without any detectable malformation (Job *et al.*, 1935). In 1950, Russell and Russell (1950) found similar results for mice and Russell coined the well-known "all-or-none-rule" in 1956, i.e. "killing or normality" (Russell, 1956, p. 378).

Since that time, quite a number of papers have confirmed the results reported by Job and Russell for rats (Hicks, 1953; Brent and Bolden, 1967; Roux *et al.*, 1983) and mice (Russell *et al.*, 1959; Friedberg *et al.*, 1973; Schlesinger and Brent, 1978; Mazur, 1984). It was generally accepted that during the preimplantation period the pluripotency of the blastomeres or the low degree of differentiation of the later stages could compensate for cell loss to a certain extent and that further radiation damage was repaired. Therefore, no malformations were expected to be induced during this developmental stage.

However, starting in 1959 first results were published that have cast some doubt on the general validity of the rule mentioned above (Rugh and Grupp, 1959, 1960): exencephalies were observed after single or fractionated exposure of murine preimplantation stages. These results provoked a lot of criticism

(for a review see, for example, Mole, 1992). The major points of this criticism were the lack of a clear dose-response relationship and of sound control data.

During the subsequent years, however, further information was obtained. Thus, Ohzu (1965) observed a marked increase in the frequency of polydactyly of the forefeet of mice after radiation exposure on days 0.5 or 1.5 after conception; he, however, attributed these malformations to indirect effects.

In 1988, an increased frequency of malformed fetuses on day 19 of gestation was observed after exposure of zygotes 1 h after conception with either X-rays or neutrons (Pampfer and Streffer, 1988). Most frequently, gastroschises were found in these studies with the mouse strain "Heiligenberger Stamm"; this type of malformation occurs at a comparatively high frequency in this strain also in the controls (around 1% at the time of the study). The observed increase was very pronounced (about 20% malformed of all surviving fetuses after 2Gy of X-rays or 0.75 Gy of neutrons), clearly dose-dependent and significantly different from the concurrent controls (as mentioned, about 1%). Basically similar results were reported by the group of Generoso (Rutledge *et al.*, 1992), although the types of malformations were different and the frequency of malformed fetuses less pronounced after radiation exposure of (C3HxC57Bl)_F₁ mice.

The capability of the induction of malformations during the preimplantation period in some mouse strains is not restricted to ionizing radiation. Quite a number of chemicals (e.g. N-methyl-N-nitrosourea, ethylene oxide, ethyl methanesulfonate, diethyl sulfate, dimethyl sulfate) are able to induce malformations after

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application during early embryonic stages (Bossert and Iannaccone, 1985; Generoso *et al.*, 1988; Rutledge *et al.*, 1992). Whether the mechanisms of induction are comparable for all agents under study is not clear in the moment.

In the following, we would like to summarize the data of our group with regard to the induction of malformations after radiation exposure of murine preimplantation stages. In particular, we will try to address the question of the mechanism that is responsible for the malformations under these conditions. This mechanism must be different from that one which is most frequently discussed, when one is talking about malformations induced during organogenesis: killing of critical cells in the organanlagen. Cell killing cannot be the relevant mechanism for the induction of malformations after radiation exposure in the zygote stage. As only one genome is irradiated in the zygote from which a malformed fetus develops, it is tempting to assume that this malformation process has a genetic background. Therefore, comparative studies have been carried out with zygotes of a second mouse strain and with zygotes after cross-breeding both strains. In addition, cytogenetic damage and protein expression have been analysed.

Results

Radiation exposure of zygotes

There are two important aspects associated with radiation exposure of Heiligenberger embryos on day 1 of gestation: Firstly, there is a statistically significant increase in the number of malformed fetuses of the Heiligenberger mice with radiation dose after exposure to X-rays or fast neutrons (Fig. 1), and secondly, the extent of this effect changes within a few hours (Fig. 2). The latter aspect will play some role in the discussion of indirect effects.

Radiation exposure of preimplantation stages beyond the zygote stage

An increase in the number of malformations after radiation exposure is not restricted to the zygote stage. All preimplantation stages do show a certain probability to react with malformed fetuses after radiation exposure (Müller and Streffer, 1990). The sensitivity, however, is reduced when one compares the data with the results obtained after irradiation on day 1.

The threshold question

The results obtained after radiation exposure of zygotes and of later preimplantation stages offer the unique possibility to test the assumption that radiation dose-response curves of processes that require damage to only one cell do not show a threshold dose, whereas in the case that damage to several cells is necessary a threshold has to be expected (Hulse and Mole, 1982).

The data presented in Figure 3 reveal, that indeed after exposure of 1-cell embryos there is no indication of a threshold dose, whereas exposure of 32- to 64-cell embryos clearly goes with a threshold dose. The latter result indicates that in the multicellular situation it is not sufficient to damage one cell. Obviously, other cells can compensate for such a type of damage; only after exceeding a certain threshold, this compensation is no longer working, because too many cells have been damaged. This

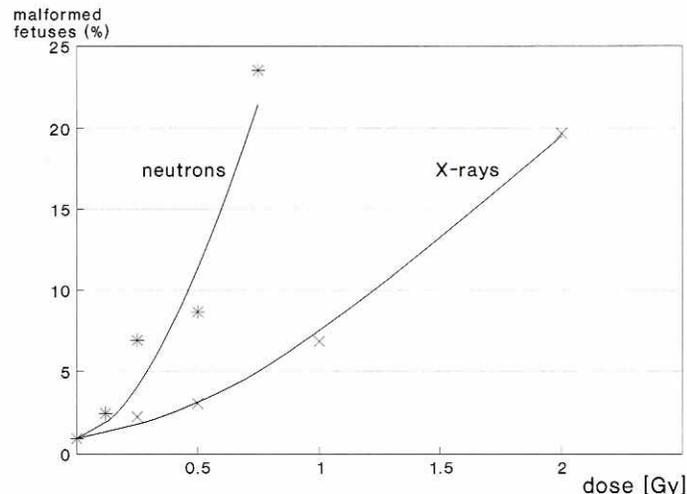


Fig. 1. Dose-response relation of the induction of malformations after exposure of zygotes (1 h post conception) to neutrons or X-rays (data from Pampfer and Streffer, 1988).

requirement also explains, at least partly, the lower sensitivity of stages beyond day 1 of murine development.

Comparison of the types of malformations induced after exposure of zygotes or during organogenesis

An important aspect that has not been addressed up to now shall be touched on in the following: the almost exclusive type of malformation observed after exposure of preimplantation stages of the Heiligenberger strain was the gastroschisis. Figure 4 shows a fetus with such a gastroschisis. This fetus is somewhat unique in so far as it belongs to the rare cases in which concomitantly to a gastroschisis also an exencephaly was observed.

Figure 5 reveals the difference in the malformation spectrum after radiation exposure of zygotes or of embryos during organogenesis. Whereas gastroschisis dominates after exposure on day 1, this type of malformation is clearly less prominent after exposure on day 8; in this latter exposure condition, the proportion of exencephalic fetuses has markedly increased.

Strain dependence of the results and cross-breeding experiments

From the beginning, we assumed that the effect observed was strain dependent. In order to test this assumption, we repeated the experiments using C57Bl mice (Müller *et al.*, 1996). Indeed it turned out that C57Bl mice did not respond with an increase in malformations after radiation exposure of preimplantation stages (1.7% in the controls, 0% in the 1 Gy group), whereas exposure during organogenesis resulted in the expected augmentation of the number of malformed fetuses (almost 60%). Actually, radiation response of C57Bl mice during organogenesis was even more pronounced than that one of Heiligenberger mice.

Cross-breeding of Heiligenberger and C57Bl mice resulted in F₁zygotes that more or less did not show an increased risk to acquire a malformation after radiation exposure of this stage. The "more or less" refers to the result that in the case of Heiligenberger mother and C57Bl father a marginally significant

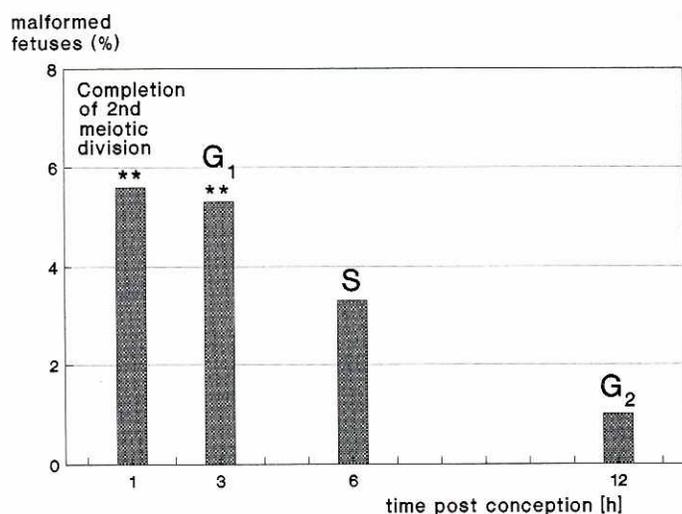


Fig. 2. Induction of malformations in various cell cycle stages of the zygote with X-rays (1 Gy; data, which are corrected for the controls, from Müller and Streffer, 1990; **significantly different from the contemporary control at $P < 0.01$).

increase was observed (controls: 0 malformed fetus among 162 survivors, i.e. 0%; 1 Gy exposure: 4 malformed fetuses among 128 survivors, i.e. 3.1%). The significance of this result was simply due to the very unusual observation that no malformed fetus occurred among 162 control fetuses. This was different for cross-breeding of C57Bl mothers and Heiligenberger fathers: controls with 1 malformed fetus among 222 living fetuses (0.5%), 1 Gy group with 5 malformed fetuses among 190 living fetuses (2.6%). For comparison: Heiligenberger showed 2.6% malformed fetuses in the controls and 13.6% after a 1 Gy exposure of the zygote.

Thus, the pronounced sensitivity to respond to radiation exposure of the zygote with an increased number of fetuses with gastroschisis is specific for the Heiligenberger genome.

Chromosomal aberrations in skin fibroblasts of normal and gastroschisis fetuses

If the genetic background is so important one should expect changes of the genome that are related to the occurrence of malformations. As gastroschisis manifest between days 15 and 17 of murine gestation, it is at least difficult to study the genome early in gestation for radiation-induced modifications, because there is no indication whether the fetus analyzed will be normal or malformed. Therefore, we decided to score chromosomal aberrations in cultured skin fibroblasts of day 19 fetuses. The results have been published in detail (Pampfer and Streffer, 1989); the most important aspects will be summarized here.

Skin fibroblasts of normal control fetuses showed 8.25 aberrations per 100 metaphases (5.5% of all metaphases were aberrant), non-malformed fetuses, which had been irradiated in the zygote stage, revealed 20.5 aberrations (12.1% aberrant metaphases), and fetuses with gastroschisis as a consequence of zygote exposure had 25.1 aberrations per 100 metaphases (17.3% aberrant metaphases). This result is remarkable for two reasons. On the one hand, skin fibroblasts of fetuses whose cells were radiation exposed many cell generations earlier (in

the zygote stage) showed significantly more chromosomal aberrations than unirradiated fetuses. On the other hand, there is also a difference between radiation-exposed non-malformed and gastroschisis fetuses. The latter ones had a significantly higher frequency of aberrant metaphases when compared with the non-malformed, but irradiated fetuses.

There was no indication that a specific type of chromosomal aberration occurred with a higher frequency in cells of malformed fetuses. Obviously, however, there was a "labilization" (increased instability) of the genome in cells of day 19 fetuses after radiation exposure of 1-cell embryos; this instability was most pronounced in cells of malformed fetuses.

Protein patterns of normal and gastroschisis fetuses

Results comparable to those ones described for chromosomal aberrations were obtained for the protein patterns of liver cells of day 19 fetuses (Hillebrandt and Streffer, 1994a). Again, no specific, that is, gastroschisis related changes were found in liver cells of radiation exposed fetuses. But in those fetuses with gastroschisis there was an approximately twofold increase of abnormal protein patterns when compared to non-malformed fetuses that had been irradiated 1 hour after conception.

Among the 25 radiation-induced gastroschisis with modifications of proteins in liver, there were 4 fetuses with a diminished intensity of one specific protein (a phosphocytokeratin). In all 4 fetuses, this protein was expressed at a reduced rate not only in liver, but also in skin and kidney. This points to a mutation of the gene early in development.

Meanwhile, Hillebrandt and Streffer (1995) reported on three specific protein changes in skin proteins of gastroschisis fetuses. In six irradiated fetuses with gastroschisis and three fetuses with spontaneous gastroschisis, one glycoprotein consistently showed a reduction of 2.9 kDa in molecular weight, whereas no such change was observed in 10 control fetuses without gastroschisis (six unirradiated, four irradiated). Similarly, the gastroschisis fetuses revealed two proteins with an increased phosphorylation compared with the non-gastroschisis fetuses.

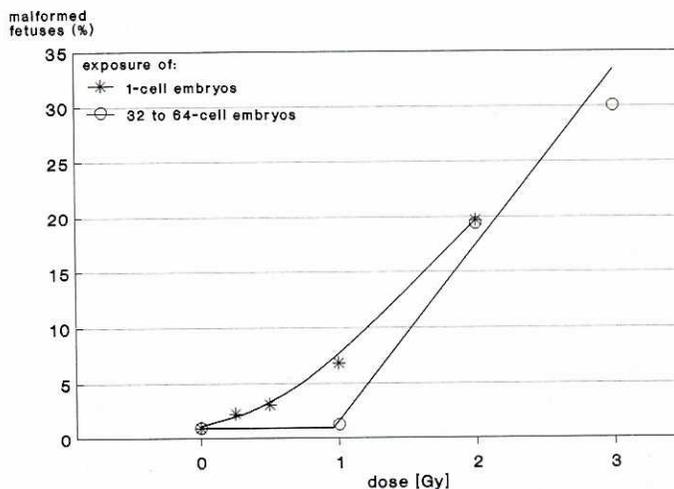


Fig. 3. Dose-response relation of the induction of malformations in 1- and in 32- to 64-cell embryos (data from Müller et al., 1994).



Fig. 4. Fetus showing a gastroschisis and an exencephaly after 3.5 Gy on day 4 of gestation.

Discussion

Our studies have shown that,

- it is possible to increase the frequency of malformations by radiation exposure of zygote and subsequent preimplantation stages in mice,
- this effect is strain dependent,
- that in particular that malformation increased in number which is present at a high frequency in controls, i.e. gastroschisis,
- the number of chromosomal aberrations was enhanced in skin fibroblasts of those fetuses that had been radiation exposed in the zygote stage and that this increase was most pronounced in gastroschisis fetuses,
- the protein patterns of liver, kidney, and skin of exposed gastroschisis fetuses showed a high frequency of modifications when compared to control- or to exposed non-gastroschisis fetuses.

These results provoke quite a number of questions which will be addressed in the following pages.

The problem of direct and indirect effects

The possibility exists that some or all of the observed malformations are due to indirect radiation effects, that is, that maternal tissue is damaged in a way preventing the proper development of fetuses. There are, however, a number of reasons arguing against such a possibility:

- The radiation doses used for the induction of malformations were in the range of 0.25 to 2 Gy for zygote exposure. These doses are too low to cause radiation sickness in mice. This is in line with the result that none of the irradiated mothers died during the experiments.
- As already outlined, sensitivity of zygote stages changes within a few hours. Radiation effects in the mother, e.g. changes of the uterine environment, will take several days before manifestation. If it were indirect effects that are responsible for the increased malformation frequencies, it is hard to see, how such a small time difference is able to evoke different effects several days later.
- In the meantime, we carried out experiments in which various stages of spermatogenesis were exposed. It turned out that also under these conditions an increase in gastroschisis frequency occurred (publication of results in preparation). In this case, indirect effects are definitely ruled out.
- Some authors have reported results after transplantation of irradiated embryos to foster mothers or of localized radiation exposure of oviducts or uterus compared with whole body irradiation (Brent and Bolden, 1967; West *et al.*, 1985a; West *et al.*, 1985b). The experiments were consistent in the finding that indirect effects on malformation induction could not be ruled out completely, that the most pronounced impact, however, came from direct effects.

Relevance of the lack of a threshold dose under uni-cellular conditions (zygote, oocyte, spermatocyte) for radiation protection

Radiation protection is comparatively easy in those situations in which threshold doses exist which must be exceeded for the induction of effects. Such thresholds have to be expected whenever it is necessary to damage many cells in order to induce the effect under study (e.g. skin burns, cataract, teratogenic effects that are caused by cell death in the developing organs; for an overview of the basic aspects see Hulse and Mole, 1982). With a few exceptions, these thresholds are in the range of about 0.5 to 1 Gy and higher.

The situation is completely different, if only one cell has to be damaged (genetic effects, and most probably tumour induction). In that case, there is always a probability greater than zero for the induction of the effect as long as energy exists that is able to carry out an ionization.

Our analysis of the dose-response relationships for the induction of malformations after exposure of zygotes (one cell!) or morulae to blastocysts (many cells!) confirms the basic assumption: no indication for a threshold in zygotes, definitely a threshold (around 1 Gy) in morulae to blastocysts. This is important for the estimation of radiation risk and for measures in radiation protection in the low dose range. These considerations are even more important for spermatocytes and, in particular, for oocytes, because in these cases the span of time at risk is much longer than for zygotes.

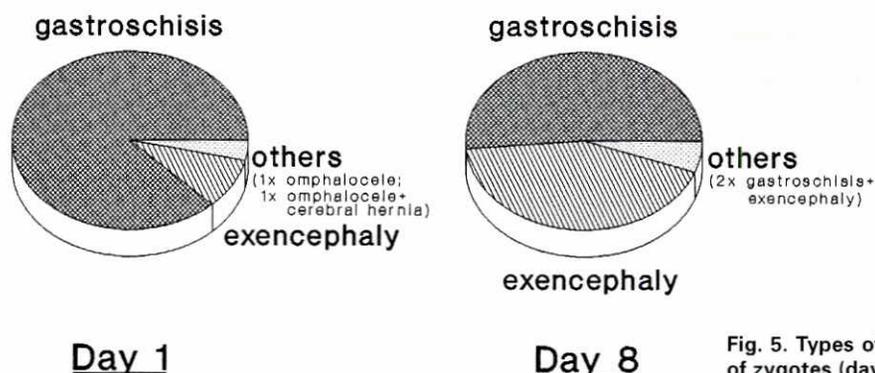


Fig. 5. Types of malformations observed on day 19 after exposure of zygotes (day 1) or fetuses in organogenesis (day 8).

Possible mechanism: genetic instability

How is it possible that radiation exposure of a single cell, the zygote, results in a developmental defect, the gastroschisis, that is observed many cell generations later? What is the mechanism behind this phenomenon?

Before we will present a hypothesis on the mechanism by which induction of malformations after radiation exposure of zygotes may be explained, we would like to summarize the facts relevant for the conclusions:

- A significant increase is observed only for such a malformation that is present in high frequency already in control fetuses. No induction of new types of malformations was found.
- Gastroschisis fetuses showed a higher frequency of skeletal abnormalities than radiation exposed, non-gastroschisis or unexposed fetuses (unpublished data).
- The induction of malformed fetuses by radiation exposure in the zygote stage was restricted to the "Heiligenberger Stamm"; neither C57Bl nor F₁-fetuses after cross-breeding of both strains showed a comparable result.
- A general increase in the number of abnormal metaphases was observed in skin fibroblasts of gastroschisis fetuses after radiation exposure of zygotes. There was, however, no specific type of chromosomal aberration.
- Protein patterns were modified at a significantly increased frequency of irradiated gastroschisis fetuses. Some of these changes were unspecific, some, in particular those in skin, were specific for gastroschisis.

The following hypothesis is an attempt to include and interpret all the results just outlined:

In the genome some weak (labile) regions or genes exist which are more susceptible to express damage induced by ionizing radiation than others. This genetic disposition results in gastroschisis with a high spontaneous frequency which is enhanced by irradiation or exposure to other toxic agents. Exposure to ionizing radiation of the early developmental stages further induces a general destabilization of the genome so that noxes acting on the fetus (e.g. insufficient nutrient or oxygen supply, infections of the mother, harmful metabolites) will be more successful in evoking detrimental effects.

It is plausible that genetics alone cannot explain the occurrence of 1 to 4% fetuses with gastroschisis in the controls. The mouse strain meanwhile used in our experiments (HLG/Zte, derived from the originally colony-bred strain "Heiligenberger Stamm") is highly inbred (more than 30 generations). The observation, that only some of the mice show a gastroschisis despite the fact that they

are virtually identical in their genomes, indicates that a non-genetic inducing agent (or several of them) is required to trigger the occurrence of this malformation and that somehow the genome must be sensitive for the action of this/these agent/s (otherwise it is not explainable, why C57Bl mice do not respond in the same way). If radiation exposure enhances this sensitivity of the genome, a higher frequency of malformed fetuses is to be expected.

Future research

The most obvious question that needs an answer refers to what is happening on the DNA level. A first step in that direction is to pinpoint the location of the genes responsible for the induction of a gastroschisis. By using chromosomal markers and cross-breeding experiments with a non-gastroschisis prone strain we might be able to identify these locations. Preliminary results show that 3 to 4 genes are responsible for these effects. Subsequently, we shall try to look for the mutation frequencies in the relevant DNA regions in fetuses derived from irradiated or non-irradiated zygotes.

The crucial genes must not necessarily be those coding for structural proteins or for enzymes. There are at least some weak indications that regulatory genes are involved, the result, for example, that gastroschisis fetuses concomitantly show a higher frequency of skeletal malformations.

This type of research may enable us finally to address the most pertinent question: What does all this mean for the human situation? Gastroschisis do occur in humans at approximately 1 per 1000 births. We do not know anything about the impact of radiation (or chemical) exposure of preimplantation stages on this malformation in humans. Without elucidating the mechanism behind the development of the gastroschisis, there is no chance to draw any conclusions for human fetuses and the population risk in general. Genetic predisposition, however, is very important in this context.

Materials and Methods

Details have been published elsewhere (Pampfer and Streffer, 1988, 1989; Müller and Streffer, 1990; Hillebrandt and Streffer, 1994a,b). In the following, only those aspects will be mentioned that are crucial for the understanding of this paper.

Mice of the "Heiligenberger Stamm" (meanwhile: HLG/Zte strain) or of the C57Bl strain were mated from 6 to 9 AM. Under our conditions, conception takes place at around 8 AM. Females with a vaginal plug were identified and radiation exposed or sham-irradiated at the times specified in the results section (day of plug = day 1). On day 19 of gesta-

tion, the uterine content was checked for the following events: early resorptions (implantation without separation of placenta and fetus), late resorptions (placenta and fetus clearly distinguishable), late fetal death (dead fetuses with macroscopically visible eyelids), surviving fetuses, fetuses with macroscopically visible malformations. For the calculation of preimplantation death see Müller and Streffer (1990). Statistical significance was tested using a chi-squared test with or without Yates' correction or Fisher's exact test, depending on the number of cases. Details of the chromosome studies have been published by Pampfer and Streffer (1989) and of the analysis of protein patterns by Hillebrandt and Streffer (1994a,b).

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