Strahlenschutzkommission

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Untersuchungen zum Bystander-Effekt, zur genomischen Instabilität und zur Rolle der Anzahl der Stammzellen bei der Leukämie-Induktion

– Schlussfolgerungen für den Strahlenschutz –

Stellungnahme der Strahlenschutzkommission mit Bericht über die Symposien vom 22./23.11.01 und 22.05.02

Verabschiedet in der 189. Sitzung der Strahlenschutzkommission am 12. Februar 2004

Inhaltsverzeichnis

Teil I Stellungnahme der Strahlenschutzkommission

1 Vorbemerkung

Für die Abschätzung von Strahlenrisiken und die Ermittlung von Dosis-Wirkungsbeziehungen im Bereich kleiner Dosen sind Kenntnisse über die molekularen und zellulären Wirkungsmechanismen durch ionisierende Strahlung von zentraler Bedeutung. Seit einigen Jahren sind unterschiedliche molekulare Effekte beobachtet und weiter untersucht worden, die das gegenwärtige Verständnis über Wirkungen im niedrigen Dosisbereich wesentlich erweitern könnten. In jüngerer Zeit werden vor allem der „Bystander-Effekt“ und die „Genomische Instabilität“ in diesem Zusammenhang intensiv diskutiert. Die Mechanismen, die diesen Effekten zu Grunde liegen, sind noch weitgehend ungeklärt, und auch ihre Auswirkungen auf die Form der Dosis-Wirkungsbeziehungen bleiben abzuwarten.

Der Begriff „Bystander-Effekt“ beschreibt die Beobachtung, dass nicht nur Zellen, die von Strahlung getroffen werden, also Energiedepositionen erhalten, Schäden aufweisen, sondern dass zusätzlich auch in *nicht* getroffenen Zellen, in den sog. „bystander cells“, ebenfalls Schäden gefunden werden.

Unter „genomischer Instabilität“ versteht man, dass nach einer Exposition gegenüber Strahlung wie auch gegenüber anderen Noxen in den Nachkommen exponierter Zellen viele Zellteilungen später Effekte beobachtet werden, die man in diesem Ausmaß nur kurz nach der Exposition erwartet hätte (Veränderungen von Genexpressionen, Chromosomen-Veränderungen, Zelltod u.a.).

Die Strahlenschutzkommission (SSK) sah bereits frühzeitig die Notwendigkeit, sich mit diesem Themenkomplex näher zu beschäftigen. Am 20./21. Januar 2000 fand eine Klausurtagung der SSK zur „Bedeutung der genetischen Prädisposition und der genomischen Instabilität für die individuelle Strahlenempfindlichkeit (Konsequenzen für den Strahlenschutz)“ statt, deren Ergebnisse publiziert wurden [1]. Am 22./23. November 2001 wurde dann gemeinsam mit dem Arbeitskreis „Strahlenbiologie-Strahlenwirkungen“ des Fachverbands für Strahlenschutz und dem Ausschuss „Strahlenrisiko“ der SSK ein zweitägiges Symposium „Untersuchungen zum Bystander-Effect und zur genomischen Instabilität“ in Bonn durchgeführt. Im Verlauf der Diskussionen zu diesen Effekten geriet die Frage nach der Rolle der Anzahl der Stammzellen bei der Leukämieinduktion zunehmend ins Zentrum der Erörterungen. Stammzellen sind nicht differenzierte, sogenannte omnipotente oder pluripotente Zellen, deren Nachkommen zu spezialisierteren Zellen differenzieren und spezielle Aufgaben übernehmen können. Die Stammzellen des blutbildenden Systems sind ein Beispiel für pluripotente Zellen. Aus ihnen entwickeln sich alle Zellarten des Blutes (z.B. Lymphozyten, Granulozyten, Erythrozyten, Thrombozyten). Es fand am 22. Mai 2002 ein ergänzendes Symposium zu diesem Thema statt.

Auf der Grundlage der Diskussionen im Rahmen dieser beiden Symposien formulierte die SSK die vorliegende Stellungnahme.

Folgende Vorträge liegen dem nachfolgenden Bericht zu den Symposien zugrunde:

Dr. Carmel Mothersill, University of Dublin:
Radiation-induced bystander and genomic instability effects

Dr. Mark Plumb, University of Leicester
Genetic instability and radiation-induced leukaemia: Genetic and epigenetic factors
und:
Genetically determined haematopoietic stem cell numbers and susceptibility to radiation-induced myeloid leukaemia

Prof. Dr. Wolfgang Göhde, Universität Münster
Precise stem cell enumeration methods

Prof. Dr. med. Baum, Universität Hannover
Therapeutic windows in the genetic manipulation of haematopoietic cells

Prof. Dr. med. Henschler, Blutspendezentrale Frankfurt
Endogenous and exogenous regulation of haematopoietic stem and progenitor cell proliferation with special view on potential relation on the size and dynamic of the stem cell pool.

2 Stellungnahme

Untersuchungen zum Bystander-Effekt, zur genomischen Instabilität und zur Rolle der Anzahl der Stammzellen bei der Leukämie-Induktion

Schlussfolgerungen für den Strahlenschutz

Risikoabschätzungen für Zwecke des Strahlenschutzes basieren auf quantitativen Angaben des Risikos der Eintrittswahrscheinlichkeit für einen betrachteten Effekt pro Dosiseinheit in einem bestimmten Dosisbereich, den sog. „Risikokoeffizienten“. Die Risikokoeffizienten für strahleninduzierte stochastische Wirkungen werden im wesentlichen durch Auswertung epidemiologischer Studien im Bereich oberhalb einiger 10 mSv ermittelt. Um für Zwecke des Strahlenschutzes zu Aussagen im Bereich kleinerer Dosen zu gelangen, ist man auf Extrapolationen aus dem hohen Dosisbereich angewiesen. Dies kann nur unter Zuhilfenahme von Annahmen zur Form der Dosis-Wirkungsbeziehung im niedrigen Dosisbereich geschehen, die aus epidemiologischen Studien nur schwer gewonnen werden kann. Für bestimmte Endpunkte jedoch können molekular- und zellbiologische Untersuchungen sowie mechanistische Studien hierzu genauere Aussagen liefern. Die Relevanz dieser Endpunkte für stochastische Effekte ist aber in der Regel schwierig einzuschätzen.

Für den praktischen Strahlenschutz spielt die Annahme einer linearen Dosis-Wirkungsbeziehung ohne Schwelle (LNT-Hypothese) eine große Rolle. Diese Linearität ist nicht unbedingt Ausdruck eines zugrundeliegenden allgemeinen linearen Wirkungsmodells, sondern kann auch einer geeigneten Anpassung entsprechen, die es offen lässt, ob durch Überlagerung vielfältiger nicht-linearer Mechanismen und durch „Ausmittelung“ es zu einem insgesamt linear erscheinenden Dosis-Wirkungszusammenhang kommen kann. Würden allerdings ein oder mehrere molekularbiologische Effekte, die in eindeutigem und direktem Zusammenhang mit stochastischen Wirkungen stehen, im Bereich kleiner Dosen einen nicht-linearen Wirkungsmechanismus in eine Richtung (supra- bzw. sublinear) nahe legen, so hätte dies - trotz sonst unveränderter Risikokoeffizienten – gravierende Auswirkungen für die generelle Konzeption im Strahlenschutz.

Im Zusammenhang mit dem Bystander-Effekt und den genomischen Instabilitäten werden nicht-lineare Beziehungen diskutiert. Konsequenzen für den Strahlenschutz könnten sich hierdurch ergeben, wenn individuelle Risikobetrachtungen angestellt werden. Hier könnten Unterschiede z.B. in der genomischen Instabilität, beim Ausmaß des Bystander-Effekts und in der Stammzellenanzahl von Person zu Person vorliegen, wodurch deren Strahlenrisiko deutlich vom Populations-Mittelwert abweichen könnte.

Die Untersuchungen zu den genannten Effekten haben erheblichen Einfluss auf die Aufklärung der molekularen und zellulären Mechanismen von Strahlenwirkungen: Epidemiologisch können statistisch signifikante Erhöhungen des Tumorrisikos oberhalb eines Dosisbereichs von etwa 50 bis 100 mSv nachgewiesen werden. Umstritten ist jedoch die Frage, ob unterhalb dieses Dosisbereichs eine Schwellendosis für strahleninduzierte Tumoren existiert bzw. welche Kurvenform die Dosis-Wirkungsbeziehung aufweist. Da diese Diskussion vor allem auf der Grundlage von biophysikalischen Modellen und mechanistischen Erkenntnissen geführt wird, muss auch die Rolle des Bystander-Effekts und der genomischen Instabilität in dieser Diskussion Berücksichtigung finden.

Die Wirkungsmechanismen zum Bystander-Effekt und zu den genomischen Instabilitäten sind noch weitgehend unbekannt. Die SSK hält daher den gegenwärtigen Stand des Wissens für noch nicht ausreichend, um Annahmen oder Hypothesen zu Abweichungen von einer linearen Dosis-Wirkungsbeziehung belastbar begründen zu können. Derzeit wird daher auch kein Handlungsbedarf gesehen, wegen der erwähnten Effekte konzeptionelle Änderungen im Strahlenschutz oder Modifikationen bei der Grenzwertfestlegung zu empfehlen. Die SSK sieht jedoch die dringende Notwendigkeit, diese Zusammenhänge in Hinblick auf die Konsequenzen für den Strahlenschutz weiter aufzuklären.

Es wird auf folgenden Gebieten Forschungsbedarf gesehen:

1. *Bystander-Effekt:* Dieser Effekt sollte in weiteren, vor allem humanen Systemen untersucht werden, um herauszufinden, wie universell er gilt. Wünschenswert wären Untersuchungen auf der Ebene kompletter Organismen. Ebenso sollten neben Alpha-Strahlen, die häufig eingesetzt werden, um diesen Effekt nachzuweisen, auch andere Strahlenarten, insbesondere Neutronen- und Elektronenstrahlen, überprüft werden. Schließlich besteht weiterhin Forschungsbedarf in der wichtigen Frage: Durch welchen Mechanismus wird die Strahlenwirkung übertragen?

2. *Genomische Instabilität*: Interessant sind auch hier Untersuchungen zur Abhängigkeit der genomischen Instabilität von der Strahlenqualität. Entscheidend sind aber gerade im Zusammenhang mit der genomischen Instabilität Experimente zum Mechanismus, denn obwohl der Effekt jetzt seit mehr als 10 Jahren bekannt ist, ist der molekulare Hintergrund nach wie vor unklar. Vor allem die Frage nach dem Zielort (Target) müsste systematisch aufgegriffen werden (Zellkern, Cytoplasma, Membranen, Signalketten; welche Rolle spielen Translokationen und Deletionen?). Ebenso ist bisher die Frage nicht beantwortet, warum es viele Zellgenerationen dauern kann, bis sich ein Effekt bemerkbar macht.

3. *Stammzellen:* Die Testverfahren zur Stammzellerfassung müssen verbessert werden; dies gilt vor allem für die einwandfreie Identifikation der verschiedenen menschlichen Stammzell-Populationen. Welche Rolle spielen eventuell in das Mäuse-Genom integrierte Virus-DNA und die Telomerase-Aktivität bei der Leukämie-Induktion durch ionisierende Strahlung? Es sollte nach denjenigen Genen gesucht werden, die die Stammzellenanzahl regulieren. Darüber hinaus sollte der Einfluss weiterer Faktoren (Mikro-Environment, Reifegrad der Stammzellen, konkurrierende Zellen) auf die Leukämie-Induktion erfasst werden. Wünschenswert sind Experimente an ein und demselben Tierstamm.

Seit der Präsentation der Vorträge von Mothersill und Plumb wurden weiterführende Arbeiten publiziert, die Erklärungsmöglichkeiten sowohl für den Bystander-Effekt als auch für einige Fragen der genomischen Instabilität erlauben [2]. Epigenetische Modifikationen können in CpG-reichen Genregionen durch Methylierungen Gene abschalten (silencing), ohne die DNA-Sequenz zu verändern. Histonmodifikationen, wie mehrfache Methylierungen durch Methyltransferasen (z. B. SuV39h1) an Lysylresten, Acetylierungen und Phosphorylierungen, können die sterische Konformation der DNA an den Histonen verändern, was Einfluss auf das „Gedächtnis“ der zellulären Transkription hat. So kann eine Lysinmethylierung der Histone zur Bildung hochaffiner Bindungsorte am Heterochromatin-Protein 1 (HP 1) führen, wodurch es zu einer dauerhaften Abschaltung von Genen kommt [3].

Die SSK empfiehlt eine erhebliche Intensivierung der Forschung, besonders auf dem Gebiet der epigenetischen molekularen Mechanismen.

3 Literatur

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[2] R. van Driel, P.F. Fransz, P. J. Verschure: The eukaryotic genome: a system regulated at different hierachical levels. J. Cell Sci. Vol. 116, No 20, 4067-4075, 2003

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Teil II Bericht über die Symposien

Bericht über die Symposien

durchgeführt im Rahmen der
Sondersitzung des Arbeitskreises
„Strahlenwirkungen – Strahlenbiologie“
des Fachverbandes für Strahlenschutz
zusammen mit dem Ausschuss „Strahlenrisiko“ der SSK,
mit dem Ausschuss „Strahlenschutz in der Medizin“ der SSK
sowie mit der Strahlenschutzkommission (SSK)

am 22./23. November 2001 in Bonn

und auf der
64. Sitzung des Ausschusses „Strahlenrisiko“ der SSK

am 22. Mai 2002 in Bonn

1 Radiation-induced bystander and genomic instability effects

Presentation by Dr. Carmel Mothersill, Radiation and Environmental Science Centre, Dublin Institute of Technology, Ireland

Summary based on the recollection of the presentation and the discussion by Prof. W-U. Müller, Universitätsklinikum Essen

1.1 Introductory remarks

Carmel Mothersill presented an overview of her work on radiation-induced bystander effects and effects of genomic instability at a special session, held on 22 November 2001, of the German-Swiss Radiation Protection Association's Radiation Effects and Radiation Biology Working Group together with the SSK Radiation Risk Committee, the SSK Radiological Protection in Medicine Committee, and the Commission on Radiological Protection (SSK). An approximately one-hour discussion with the speaker ensued.

In the following, we present the main points of the speech, with comments *in italics*. The comments are there to provide supplementary information rather than a critical appraisal. A number of critical questions came up in the ensuing discussion, whose main points are noted at the end of this summary.

1.2 Definition of the bystander effect

The term 'bystander effect' refers to a phenomenon observed after radiation exposure, where effects are shown not only by cells exposed to radiation, but also by those demonstrably not so exposed. *(In interpreting Carmel Mothersill's findings on the bystander effect it is vital to bear in mind that there are two methods of demonstrating the effect: 1. Direct irradiation of specific cells (frequently with alpha radiation, which is technically controllable to a relatively large degree) and investigation of effects in unexposed neighbouring cells; 2. (the approach used by Carmel Mothersill) irradiation of cells, transfer of medium from the exposed to unexposed cells, and investigation of effects in the latter.)*

Any effects observed in unirradiated cells would naturally have major consequences for radiation risk models, particularly at low dose ranges. *('Low dose' – and this includes sparsely ionising radiation – does not mean that all cells receive a low dose, but that some cells receive a dose and some do not. If there is a bystander effect, even unirradiated cells would have a certain risk of damage.)*

1.3 What happens, why does it happen, and what does it mean?

1.3.1 What happens? (Phenomenology)

If unirradiated cells show radiation effects, they must in some way be receiving a signal from irradiated cells. Although this signal has not yet been identified, radiation effects have frequently been demonstrated in unirradiated cells (either ones neighbouring irradiated cells or 'exposed' by way of the culture medium from irradiated cells). Such effects include cell death, mutations, chromosome changes and cell transformations or carcinogenesis.

Bystander effects have now been observed in a range of studies. Such effects can be triggered by both high and low LET radiations. They may but need not be mediated by gap junctions. *(Gap junctions are direct intercellular channels through which molecules with a molecular weight of around 1,500 can pass; the channels can be closed to quarantine off dead cells, in which free calcium rises to dangerous levels.)* The p53 status has surprisingly little relevance to bystander effects, but genetic factors overall have a manifestly large influence.

There is evidence of the cells susceptible to bystander effects primarily being those which have a 'delayed' response. As delayed effects are also characteristic of genomic instability, there may be a close connection between bystander effects and such instability. Characteristic features of delayed effects include increased frequency of all types of mutation, changes in clonogenic cell survival, and non-clonal chromosome aberrations. *(Sometimes, one observes specific chromosome aberrations to occur more frequently after radiation exposure. This is because the aberration originally occurs in a given cell and is passed on to daughter cells; that is, it is present as a 'clone'. Of course this is only possible if the aberration is one that the cell can survive; reciprocal translocations are an example of such an aberration. Nonclonal aberrations are very diverse chromosome changes, often including ones which the cell cannot survive and which are thus said to be 'unstable' aberrations.)* Non-clonal chromosome aberrations often lead to apoptosis. Certain further characteristics are also worth mentioning. Such cell cultures often feature increased mutation frequency but, remarkably, no expansion. No specific gene mutation has yet been found, although a certain genetic predisposition can be observed. And *(something that has major relevance for radiological protection)* the induced state of instability is stable.

It is an astounding finding that there should be a lasting increase in mutation frequency but no expansion of specific mutations. This can only mean that selection does not play any part. There are various possible reasons:

- The factor responsible for the genomic instability is produced continuously *(thus continuously causing an increased mutation frequency with no preference for specific mutations)*; something similar might apply for the factor that triggers the bystander effect, which would point to a relationship between the factors triggering genomic instability and the bystander effect;
- additional environmental factors are needed to maintain the unstable state;
- we are measuring an endpoint that is not determinant of selection;
- there is selection taking place, but on a level that is not being analysed.

Looking at what has been said about genomic instability so far, it would be useful to formulate and then test a working hypothesis. This working hypothesis is as follows: what lies behind the genomic instability is not genetic, but epigenetic effects. *(The term 'epigenetic' is unfortunately used in a wide range of different meanings, usually not defined by the writer. In the strict sense – and this is the meaning used by Carmel Mothersill – it refers to all hereditary changes in characteristics not caused by changes in the DNA base sequence. A possibility often referred to in this connection is that of specific DNA segments becoming methylated.)*

1.3.2 Why does it happen? (Mechanism)

First of all, we should note that the cells receiving the 'signal' triggering the bystander effect respond very rapidly in a typical manner: the start of a two-minute calcium flux in the first ten seconds followed by the six-hour collapse of mitochondrial membrane potential and finally the sustained production of oxy-radicals. Longer-term responses are apoptosis, genomic instability and continuous induction of mutations. Interestingly, the cells that produce the signal triggering the bystander effect do not necessarily respond to that signal, and vice versa.

The identity of the signal is unknown; it is very small, cannot be destroyed by deep-freezing, but can be destroyed by heat. Possibilities include a small peptide or a small bioactive molecule. The first response that is observed after low-LET experiments is the mentioned calcium pulse, which is not unlike neuromuscular depolarisation.

The findings of the experiments to date do not speak in favour of genetic changes *(in the sense of changes in the base sequence)* playing a part, although a change in gene expression such as methylation-induced gene silencing may be a significant factor. Several experiments have supplied evidence to this effect.

There are other odd findings. For example, if the bystander effect has been triggered in one cell culture and the medium from that culture is re-used for other cells, this second population of cells will show the full bystander effect. This means either the signal is not attenuated or the unirradiated cells themselves emit another signal.

1.3.3 What does it mean? (Implications for radiation risk)

Wherever the number of hits per cell is less than one, the bystander effect might augment the effective dose. This could result in a deviation from the LNT (linear no threshold) dose-response curve. But it is not possible to predict which way the deviation will go. This depends on the cellular response, not the dose.

The bystander effect raises a number of questions in the field of radiological protection. For example, to what extent is it consistent with ICRP models, which ultimately attribute carcinogenesis to double-strand breaks (DSBs)? How to quantify epigenetic risk factors in the first place? How can principles for radiological protection be formulated given the bystander effect and the observed genomic instability? What does the bystander effect imply for the concept of 'dose' in general? These questions will remain unanswerable for the time being, but the genetic background certainly appears to play a key part.

But why does a cell go to the expense of having 'anti-order' mechanisms of the kind represented by the bystander effect and genomic instability? The explanation may lie in the fact that it provides a degree of flexibility so that cells can adapt to changing environmental conditions. That is, the unstable genome makes it possible for the original phenotype that no longer adequately meets up to current conditions to turn into a new phenotype that can better deal with these changed circumstances.

1.4 Discussion

The following is a presentation of the main points of the ensuing discussion. The points are paraphrased rather than being given verbatim.

Question: The experiments show that even very low doses trigger the full bystander effect. How is this possible, considering that this result implies natural radiation exposure ought to be enough to trigger the full effect?

Answer: It may have something to do with the dose distribution, that is, with the fact that natural radiation exposure represents a chronic exposure. But a plausible explanation has not yet been found.

Question: The bystander effect induces apoptosis but is p53-independent. How come?

Answer: There is such a thing as p53-independent apoptosis involving ATM and bax.

Question: How do AT cells fare as regards the bystander effect?

Answer: AT cells produce a massive bystander effect.

Question: What part do cytokines play?

Answer: Many people believe that one of the cytokines is responsible for the bystander effect. But experiments with TGF-β, TNF-α and other interleukins were unsuccessful.

Question: There was mention that the bystander effect is cell-type-specific. What are the factors that determine this specificity?

Answer: The genotype seems to be the critical factor. The cell type (keratinocyte or fibroblast) is not crucial.

Question: What about cell cycle dependence?

Answer: No one has done any such experiments to date, but some urgently need to be done.

Question: Is the bystander effect only triggered by ionising radiation or do UV and other agents work as well?

Answer: UV A and B trigger genomic instability; no-one has looked for bystander effects yet. Studies with chemicals are difficult with the experimental system I use (investigating factors in a medium)

Question: In the current experimental design, cells are irradiated and the medium transferred to unirradiated cells in order to look, say, for cytogenetic effects. Would it not be possible to learn more about the mechanisms if one were to irradiate the cells (above all ones with known defects) after application of the conditioned medium?

Question in reply from Carmel Mothersill: What defective genes ought one to select?

Questioner's answer: For example ones to do with apoptosis!

Answer: No one has done any such studies to date. But they would be a way of finding out, for example, whether adaptive processes arise, which might then also explain why no bystander effect is seen on chronic exposure.

Question: Isn't it astounding that the effect occurs at such low doses and yet shows saturation phenomena!

Answer: There are other examples; the bystander effect seems to be an all-or-nothing effect. This is why the concept of 'dose-response' doesn't come into it.

Question: Does the bystander effect have any in-vivo importance and if yes, is it always damaging?

Answer: It does not necessarily have to be damaging; it can also stimulate specific processes. How the organism responds apparently depends on the genotype, not so much on the dose. Bystander effects and genomic instability have been demonstrated in vivo.

Question: How is the genomic instability passed on to the next generation? It would seem to require a mutation or at least mitochondrial changes.

Answer: No evidence has been found for maternal inheritance. That rules out the mitochondria.

Supplementary answer: Minisatellite instability is passed on over several male generations. This is strong evidence of epigenetic changes in DNA (such as methylation, although this does not need to be gene-specific; it can be genome-wide methylation influencing later processes like DNA replication).

Question: The saturation effect is observed at low doses regardless of the type of radiation (alpha, beta or gamma). Does this not mean that there is no difference in RBE in the low dose range?

Answer: At low ‘doses’, one ought to completely relinquish the concept of dose and work instead with the concepts of exposure and response. That is, what is relevant is not the ‘dose response’ but the ‘exposure response’, where exposure can be anything from practically nothing to lethal.

Question: What about comparisons between normal cells of a given type of tissue and tumour cells of the same tissue, or between differently differentiated cells?

Answer: Differentiated cells seem to produce a more pronounced bystander effect than rapidly proliferating cells.

Question: What is the part played by gap junctions? The connection between the Ca flux and the bystander effect seems to point to high Ca concentrations closing the gap junctions.

Answer: Jack Little always claims that gap junctions play an important part. He and his team show that no bystander effects are to be observed in cells that lack gap junctions. But gap junctions can’t play an important part given the design used in Dublin, as the medium is used to trigger the bystander effect and the cells do not come into direct contact with each other.

Question: The postulated factor triggering the bystander effect seems to be very stable!?

Answer: Yes. You can keep it in the refrigerator for several days and in a deep-freeze for years. Also, it can still be detected in the medium a whole week after irradiation.

Question: Has anyone looked for any relationship with repair capacity?

Answer: No.

Question: If as a result of the bystander effect signals can be passed on to cells that are further removed, the question arises whether UV can trigger liver tumours.

Answer: No, I am fairly sure that there are control mechanisms in vivo. The challenge lies in finding what these control mechanisms are based on.

2 Genetic instability and radiation-induced leukaemia: Genetic and epigenetic factors

Presentation by Dr. M. Plumb, University of Leicester

Summary by Dr. U. Hacker-Klom, Prof. W. Köhnlein, Dr. M. Plumb[1]

Leukaemia is one of the major health effects following exposure to radiation. In Japan, the incidence of leukaemia rose sharply among exposed individuals with increasing proximity to the detonation centre of the atom bomb [1]. The myeloid leukaemia prevailing among humans after radiation exposure is clonal in origin, the target being haematopoietic stem cells. As there is a fairly long latency between exposure to ionising radiation and leukaemia, radiation leukaemogenesis is a multi-stage process in both humans and mice. This multi-stage process is the net effect of the accumulation of gene mutations in a single target cell over time. As genetic instability includes potentially oncogenic lesions such as chromosome translocations/gene mutations and anti-oncogenic lesions in the form of delayed reproductive death, radiation-induced genetic instability in the target pluripotent haemopoietic stem cells may theoretically contribute in vivo towards leukaemogenesis [2]. At this moment however, there is no direct evidence that radiation-induced genetic instability has any long-term consequences to human health.

All mature blood cells are derived from progenitor cells which in turn are derived from a small number of stem cells that represent about 0.01 % of white bone marrw cells. Undetermined stem cells can develop into myeloid or lymphatic precursor cells. Myeloid precursor cells go on to develop after further cell division, differentiation and morphogenesis stages into erythrocytes, megakaryocytes, monocytes and macrophages, granulocytes and eosinophils. Lymphatic precursor cells develop through several division, differentiation and morphogenesis stages into T- or B-lymphocytes. Thus, for example, an adult human being has approximately 3×10^{13} red blood cells and the lifespan of an erythrocyte is roughly 120 days. The normal erythrocyte production rate of 10^{10}/h is therefore tightly regulated and ultimately dependent on the haemopoietic stem cell. The number of stem cells is presumably predetermined during embryogenesis.

The leukaemia rate in humans is 1/10,000 per year, a quarter of which is acute myeloid leukaemia (AML). 2 % of all malignancies in humans are leukaemia. The German Cancer Atlas cites a 3 % incidence rate, which would mean leukaemia ranks ninth among all causes of death [2]. Leukaemia is the eleventh most frequent form of malignancy in the United States [3]. Unlike the majority of de novo acute leukaemias that carry specific chromosomal translocations (activated oncogenes), allele loss (5q- and/or monosomy 7) is typical of AMLs that arose in radiotherapy (and chemotherapy) patients and A-bomb survivors [4,5], suggesting that tumour suppressor gene inactivation is essential in the radiation leukaemogenic process. 5q-/-7 AML represent 10-20 % of AMLs [6], so an incidence of 1/100,000-1/200,000 per year inferred. The myeloid 5q-/-7 AMLs that arose in the about 80,000 A bomb surviors studied are therefore typical [1, 5].

Activation of an oncogene only requires a single hit, but tumour suppressor gene inactivation requires two hits. Furthermore, there is a genetic component that determines relative suscepti-

[1] updated in 2003

bility to radiation-induced genetic instability in mouse and humans [7,8]. Human genetic diseases connected with chromosome instability predispose towards haematopoietic malignancies such as Fanconi's anaemia. In mouse, inactivation of genes that control genome stability promotes the development of cancer and in particular lymphoma.

There is not a simple genetic relationship between radiation leukaemogenesis and radiation-induced chromosome instability in stem cells, as data from animal experiments show.

Dr. Plumb then reported on studies with mouse models: Depending on the genetic background, AML, a mixed-lineage pre-B lympho-myeloid leukaemia (L-ML) or thymic lymphoma are readily induced in certain inbred mouse strains by moderate doses (acute 3 Gy; 0.5 Gy/min; or fractionated 4 X 1.2 Gy at weekly intervals) of ionising radiation [9]. The average latency between irradiation at the age of 8-12 weeks and the mean onset of the disease is 18 months at a maximum life expectancy of approximately 30 months [10]. AML is indicated by the emergence of immature myeloid metamyelocytes in blood, bone marrow and spleen. Cytogenetic and loss-of-heterozygosity (LOH) studies suggest that like human 5q-/-7 AMLs, the mouse leukaemias also arise through allele loss i.e. tumour suppressor gene (TSG) inactivation [9]. However, as TSG inactivation normally requires two independent genetic events, and the lifetime incidence of AML (20-25 %), L-ML (10 %) and TL (80 %) in mouse after irradiation is high [9-11], this suggests that the number of genetic events required for transformation is small.

TSG inactivation can induce genetic instability as a delayed effect of the initiating radiation exposure and can amplify it in the clonal progeny of irradiated stem cells. A characteristic feature of radiation-induced AML found in 95 % of CBA/H mouse is alelic losses on chromosome 2, in most cases large (> 20 cM) and regarded as primary genetic lesions. 50 % of also AMLs show losses on chromosome 4 that can therefore be considered a recurrent secondary event; and suggests the allele loss is not causal. The deletions on chromosome 4 are mostly small (< 10 cM). However, the maternally transmitted chromosome 4 allele tends to be deleted suggesting that the paternal allele is inactivated by imprinting during normal development [9, 11]. The TSG inactivation by a single hit may therefore contribute towards the relative efficiency of radiation leukaemogenesis in mouse. Allelic losses are observed on chromosomes 4, 11, 12, 14, 15, 16 and/or 19 in radiation-induced thymic lymphomas in a C57BL/6 mouse genetic background.

Studies of high LET-induced chromosomal instability (chromatid-type aberrations) in inbred mouse haemopoietic stem cells have shown that high levels of genetic instability are observed in the CBA/H mouse, and much lower levels observed in the C57BL/6 mouse. As low levels are also observed in the (CBA/HxC57BL/6)F1 hybrid mouse, susceptibility to radiation-induced chromatid-type chromosomal instability is a recessive CBA/H genetic trait [8].

Fluorescence in-situ hybridisation (FISH) studies have shown an average of 2.6 clonal chromosome aberrations per patient in spontaneous human leukaemia and 6.2 in leukaemia among Japanese atom bomb survivors [5]. Persistent genetic instability has been observed in radiation-induced leukaemia (chromosomal instability) [5] and in irradiated human haematopoietic stem cells (chromatid-type instability) [7]. The genetic instability assumedly arises at an early stage of leukaemogenesis as a delayed indirect response to the initiating event. Losses on chromosomes 5q and/or 7 are characteristic of radiation-induced human leukaemia and the deletions tend to be small [4,5,12]. Dr. Plumb described mouse experiments designed to understand the genetic basis underlying susceptibility to radiation-induced AML. These studies are based on the simple premise that as AML-sensitive (CBA/H) and AML-resistant

(C57BL/6) inbred mouse strains have been identified, susceptibility to radiation-induced AML is amenable to genetic analysis. Dr. Plumb hypothesised that the genes involved will be implicated in the regulation of the target haemopoietic stem cell and/or its response to ionising radiation. The incidence of radiation-induced AML in the inbred CBA/H mouse strain (20 %) is higher by a factor of three than in the 7 % AML incidence in (CBA/H x C57BL/6)F1 hybrids, F_1 x CBA/H and F_1 x C57BL/6 backcross mice, and the F_2 intercross (F_1 x F_1) mice (10). This suggests the predisposition of CBA/H mouse to radiation-induced AML is complex, polygenic and involves susceptibility and resistance genes in both the susceptible (CBA/H) and resistant (C57BL/6) mouse strains . In contrast, as outlined above, the sensitivity of CBA/H mouse to radiation-induced chromatid-type chromosome instability is inherited recessively. Similarly, susceptibility to thymic lymphoma is a recessive C57BL/6 trait, while its resistance to radiation-induced chromosome instability is inherited dominantly [8,10]. Thus, there is no simple genetic relationship between susceptibility to low LET-induced AML or thymic lymphoma and high LET-induced chromatid-type chromosomal instability. There is every reason to believe that in outbred human populations, susceptibility to radiation-induced malignancies is probably complex and polygenic.

If radiation-induced genetic instability is causal in radiation leukaemogenesis, then, at the very least, the same type of instability should be detected in the X-ray-induced leukaemia and the X-irradiated target haemopoietic stem cell. FISH studies of mouse 3 Gy X-ray-induced AMLs have revealed high levels of clonal and subclonal chromosomal aberrations which are quantitatively similar to that found in A bomb survivor AMLs [5, 13]. Similarly, high levels of chromosome-type instability has been detected in 3 Gy X-irradiated mouse bone marrow haemopoietic stem cells [13], suggesting that X-ray-induced chromosomal instability rather than high LET-induced chromatid-type genetic instability is the relevant end-point in the leukaemogenic process.

Dr. Plumb reported that a genome wide screen for linkage between genotype and phenotype (AML) in affected (CBA/HxC57BL/6)F1 x CBA/H backcross mice had revealed an AML susceptibility locus on chromosome 1 and an AML-resistance locus on chromosome 6, and evidence of weaker linkage on chromosome 13. This work has now been published [14]. These loci are low penetrance as individually they increase the risk of AML by about 2 fold compared to the F1 hybrid. Together, the loci increase the relative risk of AML by 3 fold, and thus account for the difference in the AML incidence in the (CBA/HxC57BL/6)F1 hybrid (7 %) and the 20 % incidence in the parental inbred CBA/H strain.

The mouse AML susceptibility locus on chromosome 1 co-localised to the Scfr1 stem cell frequency regulator 1 locus which determines the frequency of haemopoietic stem cells in inbred mice [14]. This raises the logically compelling but provocative hypothesis that stem cell numbers is a risk factor in radiation-induced leukaemia. In mice, the differences in the number of bone marrow stem cells in inbred mice (DBA/2 vs C57BL/6) is reported to be as high as eleven fold, with the AML-resistant C57BL/6 strain having the lowest number. Significantly, DBA/2 mice are closely related to the AML-susceptible CBA/H mouse. The number of bone marrow stem cells may vary in humans by a factor of one hundred. Interleukin 9 directly stimulates the proliferation of haematopoietic stem cells. Human susceptibility to radiation-induced genetic instability is therefore likewise probably subject to individual variation.

In the ensuing lively discussion on the implications of his findings for radiological protection, Dr. Plumb stated that he suspected a correlation between the number of haematopoietic stem cells in peripheral blood and bone marrow.

Despite differing numbers of bone marrow stem cells, humans (and mice) have similar numbers of erythrocytes and leukocytes in peripheral blood. Lower or higher numbers of stem cells obviously do not confer a selective advantage.

Asked why experiments were only conducted at 3 Gy, Plumb said the number of animals and hence the cost in a lifetime study (3 years) was too high to take in the low-dose range relevant for radiological protection.

No gender-specific prevalence was observed in the laboratory animals.

Dexamethasone is often used in paediatric oncology to treat cancer-related and therapy-related oedema. In the animal experiments (at 3 Gy), this drug increased the incidence of leukaemia fourfold (from 20 % to 80 %).

Repeated questions as to the implications of genetic instability for radiological protection strategies were answered evasively by Dr. Plumb – there is no direct evidence that radiation-induced genetic instability plays a role in radiation carcinogenesis. However, various points of departure emerged in the course of discussion. For example, monitoring personnel should be made aware of individual radiation susceptibility and offered stem cell analysis. Insurance issues were also mentioned. The far-reaching ethical problems evinced by all of this were touched upon but not given in-depth consideration.

Finally, it was underlined once again that, providing the LNT model is valid, very low doses of even sparsely ionising radiation are capable of delivering the two hits needed for leukaemogenesis.

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Discussion

Summary of the discussion by Dr. Dr. G. Heinemann

Question: Do stem cells in the periphery correspond with those in bone marrow?

Plumb: I would expect a relationship but I don't know.

Question: On the problem of chromatid type aberrations, regarding which you said in your talk that they are not clearly related to any genetically induced instability because 50 % seem to be clonal and 50 % non-clonal: might this be a function of what stage of the cell cycle the cells are at when they are irradiated?

Plumb: Yes, this is to be assumed.

Question: Have any experiments been done at really low doses?

Plumb: No. The dose-response curve in CBA mouse has been known since the 70s. The experiments are very expensive, costing DM 75 per mouse per year. The number of mice would have to be increased by a large multiple at very low doses, while the number of instances of leukaemia would decrease. This is untenable in terms of cost.

Question: In your talk you stressed the importance of the number of stem cells as target cells. Are there any studies on stem cell number among human groups?

Plumb: In bone-marrow transplantation it is necessary to obtain a large number of stem cells, which are harvested from various donors by enrichment in the periphery via stimulation with growth factors. It is known in clinical circles that the number of stem cells differs between individual donors by a factor of up to 100. Unfortunately, these observations have not yet been published.

Question: Are there any studies on radiation-induced solid tumours?

Plumb: I only know of one study on osteosarcoma, but I am not familiar with the data.

Question: Is there a gender-specific element in AML?

Plumb: Not in our model.

Question: Is there a relationship between stem cell number and risk?

Plumb: You could classify radiotherapy patients into those who develop leukaemia and those who do not and assign the number of stem cells in each case. This is not possible at present.

Question: Would it be useful to know your stem cell count before taking up a job that involves radiation exposure?

Plumb: It would be unethical to count the stem cells because it would require a bone-marrow biopsy.

– Kopp points out that stem cell collections are made before initiation of high-dose chemotherapy or radiotherapy and that peripheral blood is used for the purpose –

To obtain stem cells from the periphery, you previously stimulate bone marrow with growth factors to release them.

But to identify differences in the stem cell count it would be useful to find the regulator gene. The number of stem cells is determined during embryogenesis. So we need to find a gene that can be identified early in embryogenesis. Once you have found the regulator gene, you then need to identify the specific genotype corresponding to a large stem cell count and another that occurs with few stem cells. If there is a simple correlation between the genotype and the number of stem cells, it is then possible to draw conclusions about the number of stem cells by performing DNA analysis on the gene. So far this is pure speculation. But if this gene existed, you would perform a DNA analysis on it before a patient was given high-dose chemo or radiotherapy. Or one could retrospectively determine in a leukaemia patient whether he had a large or small number of stem cells.

The gene ought to have specific characteristics because it already plays a part in haematopoiesis during embryogenesis. About 100 genes come into question. As we know the genome sequence, we have an idea where it could be localised. If you look at these genes, you can rule out another 80 % or so because they are connected with other functions. So about 20 genes need to be analysed. It is relatively easy in the case of mice because we have two strains, one with a large number of stem cells and the other with a smaller number. As a result there must by definition be a difference in the regulator gene between these two mouse strains. So we

need to identify the two different genes and then prove that their function is sufficient to determine the different number of stem cells.

Question: The candidate gene in mouse, does it have a human homologue?

Plumb: Yes, the human and mouse genomes have the same number of genes and many homologous functions.

Question: Is it possible to differentiate individually between spontaneous and induced leukaemia?

Plumb: If tumour suppressor genes are identified, the way in which they are inactivated may differ between spontaneous and induced leukaemia; one might find more macro deletions in the radiation-induced leukaemia. At some point this may allow us to find markers that point to radiation-induced leukaemia. But for the time being we need populations, i.e. larger numbers.

Question: As the number of mature blood cells is very similar between individuals even where the stem cell count differs, are there enzymes from which the different activation of stem cells can be deduced?

Plumb: The total number of peripheral blood cells is independent of the number of stem cells. Only a subpopulation of stem cells is involved in the production of peripheral blood cells. This has been shown with marked stem cells in transplantation experiments. The related question of the part played in evolution by inheritance of different numbers of stem cells can be answered by saying one only needs a small number, a minimum of stem cells. Hence the number of stem cells is neither a selectional advantage nor a disadvantage.

Question: But does this not contradict your hypothesis that individuals with more stem cells tend more towards leukaemia – an evolutionary disadvantage?

Plumb: Firstly, leukaemia risk is low overall. Secondly, it is a disease of advanced age. That is, you have already had children before you die of leukaemia. Hence there is no selectional pressure in the population.

3 Genetic predisposition to radiation induced leukaemia – a question of target cell quality and pool size

Summary of the presentations 3.1 – 3.3 by Dr. Dr. G. Heinemann und Prof. W.-U. Müller:

Following the presentations given by Carmel Mothersill and Mark Plumb on 22 November 2001, in which they outlined their work on the subject of radiation-induced bystander effects and the effects of genomic instability, it became apparent that it would be worthwhile to revisit one of the more significant aspects involved. This came about because Mark Plumb had formulated the hypothesis that a significant factor in the risk of developing leukaemia following exposure to radiation is the number of 'target cells' or haematopoietic stem cells responsible for the specific type of leukaemia. The more stem cells there are, the higher the individual risk of developing leukaemia following exposure to radiation.

If this is correct, it could have a significant effect on individual radiation-induced risk of leukaemia as we can assume that individual differences exist as regards stem cell numbers. Mark Plumb was invited back to give a more detailed presentation on this particular subject. Additionally, three other experts from the field of haematopoietic stem cell research were invited to present their opinions on the hypothesis and the issue of counting stem cells.

3.1 Genetically determined haemopoietic stem cell numbers and susceptibility to radiation-induced acute myeloid leukaemia

Presentation by Dr. Mark Plumb, University of Leicester

What led to the hypothesis outlined above was some research conducted on two mouse strains (CBA and C57BI) with differing levels of risk of developing acute myeloid leukaemia (AML). While 12-week old CBA mice react to a single dose of 3 Gy with around 20 % AML cases over their remaining lifetime, this is not the case with C57BI mice under the same conditions. However, following exposure to fractionated radiation at 4 x 1.25 Gy, thymus lymphoma can be observed in C57BI mice if fractionation is started at a very young age (4 weeks). The same reaction is not evident in adult animals.

Linkage analyses at a spacing of around 20 cMorgan show that between four and five gene loci are involved in the development of AML, although no one gene is solely responsible. It is particularly interesting to note the involvement of Chromosome 1: linkage analyses show that the gene for the stem cell frequency regulator (Scfr1) lies in the area identified as contributing to AML development (this has now been published; Boulton et al.: *Blood* **101**, 2349-2354; 2003). Furthermore, literature contains reports in which various mouse strains have differing stem cell numbers: high in CBA mice, average in BALBc and low in C57BI.

While it is not possible to isolate primitive stem cells using a cell-surface marker, the use of additional antigens allows elimination of the various stages using a combination of different markers (lineage depletion or Lin^-) to identify specific subpopulations within the remaining cells. Research conducted by Mark Plumb's team on Lin^- populations of this type allowed

identification of long-term repopulating (or immature) stem cells in bone marrow in mice, using antibodies that targeted specific cell-surface antigens (Sca1, C-kit). It became apparent that Lin^- $Sca1^+$ $C\text{-}kit^+$ populations in CBA mice were considerably larger than those in C57BI mice. In contrast, the short-term repopulating (or somewhat more mature) stem cells that were also identified (i.e. Lin^- Sca^+ $Flk\text{-}2^+$) via the Flk 2 antigen exist in comparably large numbers in all mice strains. These results match the hypothesis that the target size, i.e. the number of immature stem cells, is a significant risk factor in triggering leukaemia. However, this hypothesis is over-simple:

a) It remains to be proven that the genetic control of the Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ bone marrow cells does indeed map to Scfr1 and the AML susceptibility locus.

b) It is not in fact the number of stem cells in a mouse that determines risk – it is the number of stem cells that *survive* the 3 Gy leukaemogenic dose of X-rays.

These points are currently being addressed (M. Plumb, September 2003).

This raises the question as to whether this applies to humans as well. The problem is that despite intensive research, it has not been possible to obtain quantitative data on stem cell numbers in humans.

3.2 Therapeutic windows in the genetic manipulation of haematopoietic cells

Presentation by Prof. Christopher Baum, Medizinische Hochschule Hannover

In his opening speech, Christopher Baum praised Mark Plumb's hypothesis as an interesting notion that was supported by numerous findings. But as in Popper's theory, a hypothesis can only be refuted and never proven, the intention in Baum's presentation was to pinpoint any weaknesses as well as any potential need to modify Plumb's hypothesis.

Firstly, Baum addressed the question of whether there are any differences between leukaemia in mice and in humans, and if so, what they might be. There is much evidence for the fact that fewer hits are necessary to trigger leukaemia in mice than in humans. In experimental mouse systems, two hits have proven sufficient: one in the apoptosis path and one concerning cell proliferation/cell differentiation. A significant difference between mice and humans involves telomerase activity, which is much more finely regulated in human cells than in rodent cells, the outcome being that rodent cells can be more easily transformed than human cells. Stem cells usually have high telomerase activity. While it is reduced in differentiated human cells, it remains relatively high in rodent cells.

The pathology of leukaemia is similar in mice and humans, but the temporal processes are naturally very different. While we have to think in terms of years and decades for humans, it is a matter of months where mice are concerned. One significant difference between mice and humans is that in mice, virus DNA integrated into the genome plays a significant role and can be activated via exogenous factors (e.g. radiation). While such viral DNA segments are also contained in the human genome, there is no evidence of activation in relation to leukaemia in humans.

What is also important is the fact that mice have a significantly higher cell turnover than humans. This means that the life of a single cell is considerably shorter. The question also arises as to whether stem cells in mice are actually the target cells in triggering leukaemia. At least in C57BI mice, there is a suspicion that the thymus lymphoma observed following irradiation can be traced to activation of an integrated retrovirus in the genome. In a CBA mouse, no such integration of a retrovirus in the DNA is known. Thus, just as in humans, in the case of a CBA mouse it may involve the accumulation of hits in the genome of the cells in the stem cell store.

Of key importance are the testing procedures that aid identification of stem cell numbers. The traditional procedure involves the spleen colony test, which exploits the ability of stem cells to form colonies on the spleen surface of a heavily irradiated mouse after injection. This does not involve the actual stem cells but rather late progenitor cells. Today's method involves measuring stem cell function using repopulation testing procedures. In this way, a correlation has been identified between repopulation ability and specific antigens on the cell surface. But the correlation is not an exact match and it is thus important not only to determine the antigen profile, but also to show that cells with a specific antigen profile act like stem cells, i.e. they are able to repopulate bone marrow.

Another question concerns whether the pool size is the single factor in the induction of leukaemia from radiation. Experiments using a retrovirus vector can provide more detailed information. This approach has the advantage that very specific cells contain the retrovirus and no exchange of the retrovirus between the cells can take place. By adding a second artificial gene, these cells were marked and transplanted into mice whose bone marrow had been destroyed through exposure to radiation. After seven months, no signs of leukaemia could be found. The stem cells were removed and transplanted into a second group of mice whose bone marrow had also been destroyed. A year after the first transfusion, leukaemia manifestation was found in various stages, right up to a fully developed acute myeloid leukaemia. Further analysis showed that one clone was responsible for the various stages of leukaemia in the receiver mice. The place at which the retroviral DNA was integrated was also identified, as was the fact that the second gene inserted also played a role.

It must be noted that although one clone was identified, very different leukaemia manifestations resulted and this means very different latency periods. How can this be explained? We need to imagine that it involves not only the malignant clone, but also the surrounding, non-malignant competing cells. Thus, expansion of leukaemic cells takes longer due to competing cells, which in turn naturally allows for further genetic changes. It can thus prove interesting to stimulate stem cell expansion (e.g. through induced bleeding, use of specific medication or through transplantation to other animals).

What does this mean in terms of how the pool size influences leukaemia induction? The results of the aforementioned experiments make it probable that apart from the number of stem cells, the number of competing cells is also a significant influencing factor. The fewer competing cells, the faster malignant cells can expand. If there are many competing cells, expansion is considerably slower. And consideration must be given to the differences in individual systems. In C57BI mice, stem cell pool size in young animals is relatively small and increases with age so that the number of potential competing cells also increases with age. By way of contrast, in CBA mice the pool size of competing cells decreases with age. We know little about the situation in humans.

In sum, Baum determined that the hypothesis of stem cell pool size significantly influencing leukaemia induction is well supported by a number of existing findings. Other influencing factors (like the number of competing cells) should, however, be taken into consideration. It would certainly be desirable to test the significance of the individual factors in one and the same mouse strain and not by comparing different strains. This can be achieved through targeted external manipulation of pool sizes (through specific chemicals, induced bleeding, etc.).

3.3 Endogenous and exogenous regulation of haematopoietic stem and progenitor cell proliferation with special view on potential relations to the size and dynamics of the stem cell pool

Presentation by Prof. Reinhard Henschler, Blutspendezentrale Frankfurt

Reinhard Henschler's presentation focused on two key questions:

1. How can stem cells be measured?

2. How is the stem cell pool regulated?

A characteristic of immature, not-yet-determined stem cells is the surface antigen CD34. However, the stem cells that interest us in terms of leukaemia induction make up only a small section of this population. One way of identifying these cells is to allow a feeder layer (e.g. on non-activated fibroblasts) to grow as a bone marrow stroma substitute – in other words, to grow a long term bone marrow culture. After a while, small 'foci' (groups of daughter cells) grow wherever stem cells exist (given their microscopic picture, these structures are also defined as CAFCs, for cobblestone area-forming cells). DBA mice, for example, show a large number of CAFCs within the first five weeks of culture, while other mouse strains show considerably lower numbers.

Another test procedure, long term bone marrow culture initiating cell assay (LTC-IC), which uses six and eight-week cultures, produces similar results although a number of cells also grow that do not belong to the actual stem cell pool but rather to the progenitor cells.

A range of chemicals can be used to modify stem cell numbers. The chemicals show different toxicity patterns for different stem cell types: 5-fluorouracil, for example, is highly toxic to marginally determined stem cells and has hardly any impact on numbers of primitive, non-determined stem cells. In contrast, Busulfan has the opposite effect. Thus, the chemicals have a different effect to ionising radiation because the latter reduces numbers in all stem cell types.

These in-vitro test procedures only identify proliferation activity in stem cells and do not testify to the quality of those cells. Thus they do not answer the question of whether the stem cells fulfil their stem cell function. This can only be done via in-vivo testing, which checks whether, after complete destruction of the body's own stem cells, transfused stem cells are responsible for creating cells that circulate in peripheral blood. In DBA mice, this testing procedure shows considerably faster regeneration of peripheral cells than in C57BI mice, which in turn is evidence of a higher stem cell number in DBA mice.

There is much evidence to support the fact that stem cell numbers are regulated and influenced by a variety of factors (growth factors, medication). Of particular importance in this regard are procedures that aim to expand stem cell numbers, whereby a higher number of stem cells in also found in peripheral blood. Naturally, this considerably simplifies stem cell transplantations (which are more stem cell transfusions). This is made possible, for example, by G-CSF (granulocyte colony stimulating factor). G-CSF not only increased stem cell numbers in humans, it also increases their release into peripheral blood.

As outlined above, it is not possible to isolate primitive stem cells using a cell-surface marker, but it is possible to eliminate all differentiated stages via a combination of different markers (lineage depletion or Lin^-) and then to identify the C-kit^+ Sca-1^+ cells in the remaining cells. In this way, it is possible to enrich primitive stem cells by up to one primitive stem cell in about 40 cells instead of the usual one primitive stem cell in about 100,000 cells.

There are also endogenous factors that influence stem cell numbers. Of particular importance in this regard is the microenvironment, i.e. the stroma in bone marrow. This microenvironment can be tested using a procedure known as CFU-F (colony-forming unit fibroblast), which clearly highlights the importance of the stroma. That means that the microenvironment may also be a significant influencing factor in radiation-induced leukaemia.

3.4 Precise stem cell enumeration methods

Presentation by Prof. W. Göhde, Universitätsklinikum Münster

Summary by Prof. W. Köhnlein

Prof. Göhde started his talk on Precise Stem Cell Enumeration Methods by considering the significance of knowing the absolute number of stem cells. The flow cytometry method he has developed is a volumetric count and thus a determination of concentration in which a volume is ascertained simultaneously with the number of microparticles contained within that volume. Göhde showed that lysis of human blood and bone marrow samples depends not only on the buffer used, but also on the donor and previous treatment undergone by the donor.

Because in other common methods of stem cell enumeration, up to 40 % of the stem cells (CD 34+ and CD 45+) are lost during lysis performed to eliminate erythrocytes, the method presented by Göhde offers a significant advantage in doing without the lysis step.

In the ensuing discussion, Dr. Plumb emphasised that he likewise does not use lysis when ascertaining stem cell counts and that variations in stem cell number between different mouse strains is manifestly not due to error caused by lysis.