Final Technical Report
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U.S. Department of Energy

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"Responses of Cell Renewal Systems to Long-term Low-Level Radiation Exposure"

A Feasibility Study Applying Advanced Molecular Biology Techniques on Available Histological and Cytological Material of Exposed Animals and Men

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Introductory Remarks

In the concluded feasibility study (DE-FG02-02ER63293) Dr. Meineke and his group investigated the valuable stored material of irradiated dogs in the ANL experiments for further use regarding recognition of effects of low dose rates of low-LET ionizing radiation. Therefore, this material was checked for condition, availability and suitability for cell and molecular biology methods. It was not the intention to provide essential data on the mechanisms involved in tissue damage following chronic irradiation. Nevertheless extremely promising data emerged on specific effects of low dose rate radiation on the immune system, which have not been reported before.

1. Condition of the Material (Dog Tissues) Received for the Study

In the time from the end of January 2002 to February 2003 100 formalin-fixed, paraffin-embedded tissue blocks were examined from a total of 28 dogs. These belonged to four irradiation groups (3 mGy/day, 7.5 mGy/day, 8 mGy/day, 18.8 mGy/day or 37.5 mGy/day plus unirradiated controls). Most of the material (more than 90%) was lymph nodes. There were only three specimens of spleen and two of skin. Fresh dog tissue was obtained from the University of Munich for comparison purposes.

The received material strongly varied regarding the condition and embedding. Obviously, the protocols for paraffin-embedding had been changed throughout the long duration of the ANL experiments. It was not possible for the investigators to attach the poorer material to certain time periods. In a first step the material was divided into three groups and cataloged. In figures 1 to 3 tissue blocks from the three different groups are illustrated.

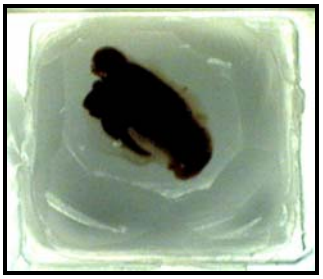

Figure 1

Figure 2

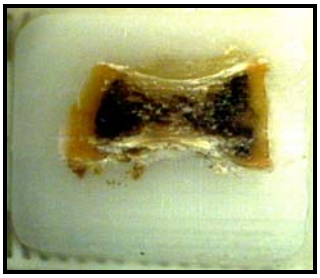

Figure 3

Figure 1: Well preserved paraffin block of group 1
Figure 2: Paraffin block of group 2 with multi-embedding in poor condition
Figure 3: Paraffin block of group 3 (bone marrow) in extremely bad condition

Most of the blocks of group 1 could be used for immunohistochemistry. Sufficient thin tissue sections could be prepared from 46 out of 55 blocks of group 1 (83%). Group 2 has only been exemplarily used as most of this material had become flakily dry. The problem with this group is that up to three different organs are embedded within one block. Because of size and difference in tissue fragility it is impossible to prepare thin sections from this material appropriate for immunohistochemistry examinations. Group 3 blocks could not be used for study because of inadequate tissue quality. In summary, it was concluded that only about 50-60% of the embedded dog tissues can be used for future research.

According to Dr. Tom Fritz there are about 5200 blocks filed by dog number and about 4700 blocks grouped by number with various dogs having been processed at the same day. In

addition, more than 54000 tissue slides (partly produced from the blocks) are stored in 22 cabinets at ANL. One task of a future project will be to identify relevant material for further scientific evaluation.

2. Methods Applied

The evaluation of the material was started with the morphological examination for pathological changes. Therefore, tissue sections were stained with hematoxylin and eosin. In some cases special staining procedures (Giemsa, PAS and Turnbull) were applied.
Simultaneously immunohistochemical evaluation was performed, using the APAAP (alkalic-phosphatase-anti-alkalic-phosphatase)-method, which allows a binding of enzymes to monoclonal antibodies. According to the method of Cordell et al (1984) various primary antibodies (ICAM-1, CD68 (macrophages), CD29, CD49f, CD49b (all integrins with a β1-chain) were used. Sixteen other antibodies including cytokines were tested, but did not prove to function reproducibly. Counterstaining of tissue sections was performed using Mayers hemalaun-solution. PCNA staining was performed according to the manufacturer´s instructions.

DNA-fragment-detection (TdT-Assay) used the ApopTaq Plus Kit (Oncor, Appligene, Heidelberg) as described recently (Meineke et al 2002). This method allows the detection of both single and double strand breaks *in situ*. The kit was used as recommended by the suppliers. Densitometric measurement of the averaged signal of the micronuclei in comparison to the corresponding nuclei was performed using image analysis (Kontron).

Target sequences for nested RT-PCR were the adhesion molecules ICAM-1, VCAM-1, CD44 and the cytokines IL-1β, IL-6, TNF-α, TGF-β. For gene expression analysis, total cellular RNA from canine tissues had to be isolated first. Whereas RNA extracted from paraffin-embedded tissues is known to be often of poor quality, there are no studies available discussing the extent of RNA degradation in old paraffin material. To overcome this drawback, measurement of the degree of degradation was performed with part of the blocks according to a method specially developed in the Institute of Radiobiology (Staßen et al. 2003 in press). In short, this method uses effects of GAPDH-RNA degradation within tissues of various well-defined degrees of degradation normalized to certain other housekeeping gene expressions. Degradation of RNA can occur before, during or after the formalin fixation process. Moreover, formalin fixation causes a cross-linkage between nucleic acids and proteins and covalently modifies RNA by the addition of mono-methylol groups to the bases. It was disadvantageous that no information about the fixation and embedding procedure of the canine materials was available. Therefore, several RNA isolation methods had to be applied and evaluated for their suitability to obtain intact RNA from the canine tissue blocks. Certain modifications to current protocols had to be included. Digestion of canine materials with proteinase K followed by phenol-chloroform extraction of RNA using the Paraffin Block RNA Isolation Kit (Ambion) was finally found to be the method of choice. The derived PCR products were purified and used as templates for a second round of amplification with a second pair of sequence specific primers. The nested PCR products were analyzed by agarose gel electrophoresis. Under optimal conditions, sequence specific primers for PCR analysis bind to different exons of a given cDNA sequence. The use of such intron-spanning primers allows differentiation between amplified cDNA and possible contaminating genomic DNA both of which differ in the intron length. To design specific PCR primer pairs, sequences of canine adhesion molecules and cytokines of interest were obtained from the Genebank database (NCBI). The problem was that no DNA sequences and thus no information about the exon-intron structures were available. Based on only mRNA data, it was impossible to choose

primers annealing to different exons. Thus, there was no possibility to differentiate between amplified cDNA and genomic DNA. Therefore, the digestion of RNA preparations with DNase I had to be complete to avoid false positive results. In addition, controls using RNA that was not reverse transcribed as a template for PCR amplification had to be applied. In addition, RTQ-PCR was performed after special Primer-Probe-Design (PPD) with some of the remaining probes using Gene Amp 5700 (Applied Biosystems™, Weiterstadt, Germany). Furthermore differential gene expression in canine lymph node tissues was assayed using two Gene-Array systems. One, a non-radioactive GEArray™ System in a customized form as well as with self confectioned gene targets (GEArray Kit, SuperArray Inc., Bethesda, MD) (Meineke et al 2003, in press). As no GEArray for analysis of canine gene expression was available, the human GEArray kit was used. The array consists of two identical positively charged nylon membranes on both of which more than 20 cDNA fragments of genes of interest have been immobilized in duplicate. The cDNA probes are synthesized from two total RNA samples and hybridized to the cDNA fragments spotted on the membranes. The other Gene Array was a 12 k human gene-expression array (BD Atlas™ plastic microarray, Clontech, Heidelberg) with 12.000 gene targets. Isolation of DNA from canine tissues was performed using the DNeasy™ Tissue Kit (Qiagen, Germany) according to the manufacturer´s instruction.

Telomer-FISH was performed by Dr. K. Greulich-Bode (Div. Genetics of Skin Carcinogenesis, German Cancer Research Center Heidelberg) in collaboration with Dr. V. Meineke. The method was applied as described previously (Vazquez et al. 2002). It has been shown, that oxidative stress and /or ionizing radiation results in the accumulation of single-stand breaks in telomeres (in comparison to non-telomeric DNA), an effect which leads to accelerated telomere shortening in vitro (Petersen et al., 1998; von Zlginicki 2002).

3. Results of the Morphological Evaluation

Morphological evaluation on tissue slices indicated that the lymph node tissues of the control dogs mainly revealed a physiological structure. From the morphological point of view there seemed to be a strong correlation regarding loss of follicular structure of lymph nodes between dogs irradiated with higher doses and dogs at an older age. Indirect signs of a right heart failure and consecutive portal hypertension seem to appear in form of an increased erythrocyte deposition within both dog groups. The significance of these findings has to be reexamined by reviewing the dog case histories as well as certain clinical laboratory findings of the dogs under study. Therefore clinical and laboratory case histories of the dogs at ANL are indispensable for the proposed project.

4. Results of the Immunohistochemical Evaluation

A problem associated with the immunohistochemical evaluation of the dog material was to find antibodies suitable for canine tissue. A large panel of human antibodies had to be applied and evaluated. Despite this problem, several parameters showed reproducible results in canine tissue. Interestingly, all the above listed markers showed a correlation with the immune system. Among the reproducible markers were CD68, which is specific for macrophages, and various other markers for cellular adhesion molecules. Expression of the following adhesion molecules included: ICAM-1 (the intercellular adhesion molecule-1 from the so-called immunoglobulin superfamily), the integrins CD29, CD49b and CD49f (all those integrins share the same β1-intergin subunit). Recently, it has been shown that all these adhesion molecules are modulated by ionizing radiation (Meineke et al 2002 (1-3), Cordes et al. 2002). The investigation of effects of ionizing radiation on adhesion molecule modulation is of

outmost interest as those cell surface markers despite their original task of intercellular adhesion take part in many intracellular processes induced, for instance, by inflammation, altered cell proliferation and rate of differentiation to mention only a few of them. Thus, investigation of protein cell surface expression allows one to draw some conclusions regarding even more sophisticated cellular processes like signaling either inside-out or outside-in. The close interrelationship between protein cell surface expression of adhesion molecules on the one hand and expression of corresponding genes on the other hand (Meineke et al. 2002) allows some conclusions from one level of interest (protein expression) to the other (gene expression).

The above adhesion molecules have not been described before as being modulated by low dose ionizing radiation. Evaluation of signal expression in a future project should be performed using automatic fluorescence techniques and image analysis.

In summary, the following changes of above markers in the investigated material have been noticed. The 3 mGy/d group showed a marked increase of either CD68, CD54, CD49b, CD49f and CD29 protein expression both compared to the control group (Figure 4 and 5) and to the higher irradiated group. What was most impressive is that increased signals of the markers arose mostly in the walls and nearby of blood vessels beside focally intense staining in the remaining tissue. This also suggests a radiation-induced stimulation of immune parameters, which could involve the whole organism. In tissues irradiated with the highest dose of 37.5 mGy/d there was hardly any signal for all of the above markers, and older dogs showed even at lower doses of ionizing radiation the signal to be more discrete than seen in younger dogs.

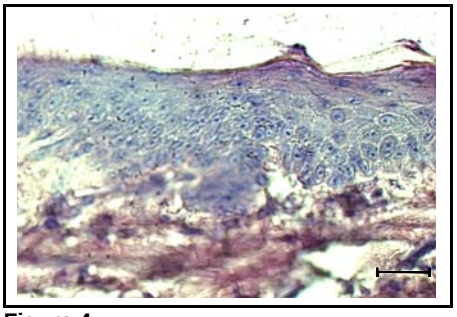

Figure 4

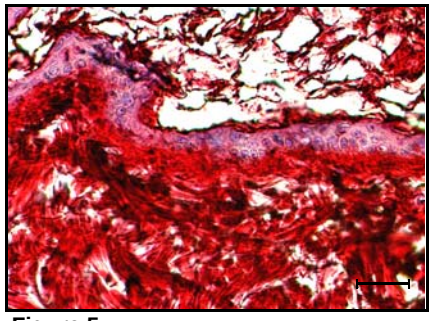

Figure 5

Figure 4: Expression of CD49f in dog skin from control group (APAAP-staining): Absence of staining, scale bar: 40 µm
Figure 5: Expression of CD49f in dog skin from the 3 mS/d group (APAAP-staining): Marked increase of APAAP-signal subepidermal, scale bar: 40 µm

This findings suggest a correlation between radiation dose, age of dogs and expression of relevant parameters for the immune system to be confirmed in a more extensive study. As the investigated parameters do not only reflect the immune system (as already mentioned above) but also relate to other processes like cell proliferation and differentiation, screening of those parameters might help to understand the effect of irradiation at different dose levels. The most striking phenomenon was that the 3 mGy/d dose group showed a significant increased signal intensity in all of the investigated parameters. This suggests an immunostimulating effect as well as a radio-adaptive response to low dose rate radiation. In addition, cell proliferation markers (PCNA) have been checked in some of the materials. Overall, a dose-dependent as well as a dose-rate dependent decrease of proliferation was noticed in the examined lymph nodes irradiated with higher dose than 3 mGy/d. Figure 6 shows proliferating cells in a lymph node from a dog from the control group and figure 7 shows a significant reduced proliferation in a tissue sample of the 37.5 mGy/d group.

5. Results of the TdT-Assay

Staining for DNA damage using the TdT-Assay was performed successfully. An increase of the incidence of TdT-positive cells was observed in tissues irradiated at a dose rate higher than 3 mGy/d (figure 8). Within the control group a low to zero range of TdT-positive cells (not shown) was detected. Within the 3 mGy/d group there was no significant increase of TdT-positive cells as compared to the tissue of the control group. If at all, there was only a focal signal of TdT-positive cells. Figure 9, in contrast, shows an increase of TdT-positive cells in the lymph node tissue of a dog irradiated with 18.8 mGy, as compared both to the control and 3 mGy/d group. Remarkably, in the group exposed to highest dose rate (37.5 mGy/d) nearly a total absence of the TdT-signal was found perhaps indicative of

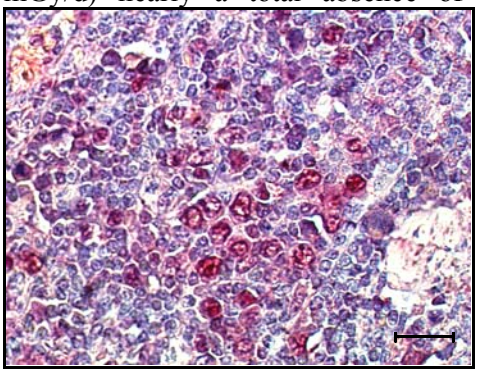

Figure 6

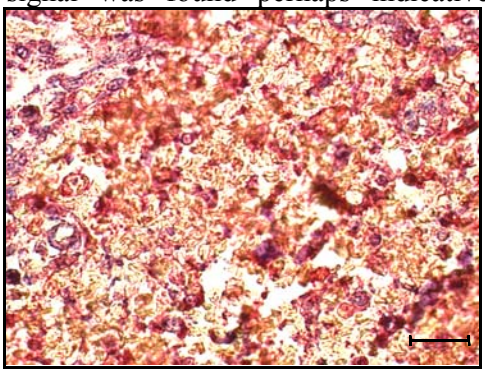

Figure 7

Figure 6: Regular lymph node histology with focal intensified proliferation (dog from the control group and APAAP-staining for PCNA positive cells), scale bar: 40 µm
Figure 7: Loss of regular lymph node architecture with a significantly reduced proliferation (dog from the 37.5 mGy/d group, APAAP-staining for PCNA positive cells), scale bar: 40 µm

necrosis similar to human lymphocytes. Other explanations for this phenomenon might be that the tissue fixation for this group of dogs was different precluding the application of the TdT-method. A third explanation might be that at higher dose rates there are either changes within the patterns of ionizing radiation-induced strand breaks which can not be detected with the TdT-method or even adverse dose effects with a relative reduction of the incidence of strand breaks occurred. These all are extremely interesting topics to be addressed in further studies. Taken together, the TdT assay proved to be suitable for the evaluation of strand breaks within the canine materials. An evaluation of a higher number of samples with the help of image analysis would allow one to find a correlations between dose rates and strand breaks observed within the tissues.

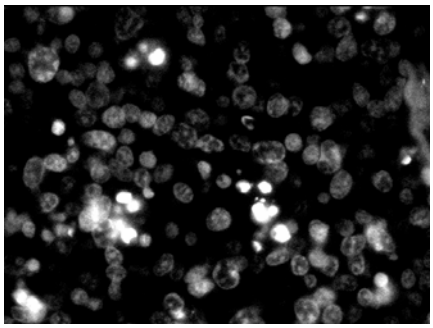

Figure 8a

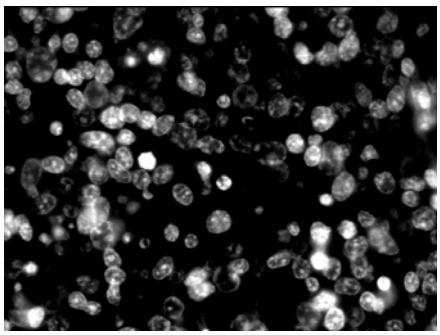

Figure 8b

Staining for TdT-positive cells with strand breaks (8a) and DAPI staining of cell nuclei of the same field per view (8b) reveals only a focally slight rate of strand breaks within a lymph node of a dog of the 3 mGy group.

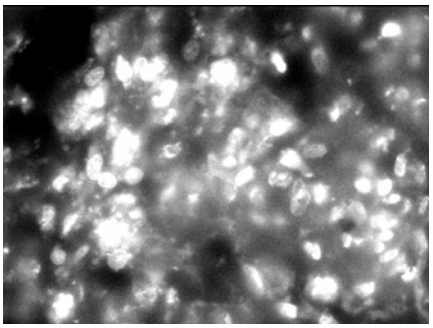

Figure 9a

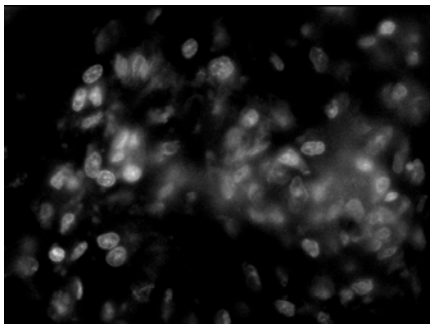

Figure 9b

Staining for TdT-positive cells with strand breaks 9a) and DAPI staining of cell nuclei of the same field per view (9b) shows areas with a high rate of TdT-positive cells (figure 9a) throughout the whole lymph node of a dog of the 18.8 mGy group.

6. Results of Measurement of RNA-Degradation

The total RNA isolated from the paraffin embedded tissue partly showed an intensive degradation. The degree varied up to $8x10^{-6}$ as compared to optimal preserved fresh dog control tissues (fixed with RNA*Later*^{TM}Stabilization Reagent, Quiagen, Germany). This variation might be due to several factors, either to varying fixation protocols, which changed within the ANL experiment or varying times from death of animals until storage of tissues. Therefore, it can be concluded that the amount of RNA to be isolated from the dog material in future studies will be extremely small. This limits all methods based on RNA-expression decisively. Despite the fact that most interesting data could be generated (see below the next paragraphs), the effect of degradation of the dog material has absolutely to be taken into account, especially when applying quantitative measurements.

7. Results of Gene-Expression-Analysis Using Nested RT-PCR

Isolation of total RNA was performed as described in material and methods. When digesting the tissues with proteinase K, some of the material was observed to be either not completely or not at all digestable due to differences in the quality of the provided material. Lymph node material from 4 dogs from the non-irradiated control group and from 4 dogs from the group irradiated with 3 mGy/d, were processed. Following RNA extraction, the samples were treated with DNase I to remove contaminating genomic DNA. Running the RNA preparations on a 1% agarose gel revealed that the RNA concentration in all samples was too low to detect

18S and 28S rRNA. Therefore, the success of RNA isolation had to be checked by PCR amplification using commercially available 18S rRNA primers. RNA preparations were reverse transcribed into cDNA using random hexamer primers instead of oligo(dT) primers. The reason was that the poly(A) tail of fixed mRNA is often modified or blocked so that oligo(dT) primers would not anneal. Before reverse transcription, RNA had to be incubated at 70°C in order to remove methylol additions in RNA. PCR products were separated on a 2% agarose gel. As shown in Fig. 10, a distinct DNA fragment with the expected size of 324 bp was amplified from each cDNA sample. The successful amplification of 18S rRNA indicates that an appropriate method could be established to isolate total cellular RNA from canine tissue blocks evading the difficulties usually associated with such approach. Before performing nested RT-PCR on the paraffin embedded material, designed primers and PCR conditions were tested for suitability using fresh canine skin and lymph node tissues. Total RNA was isolated using the RNeasy Kit which worked well using the canine material. The integrity of the 28S and 18S rRNA as a sign of intact total RNA was verified by agarose gel electrophoresis. All adhesion molecules and cytokines of interest could be amplified using reverse transcribed RNA from fresh canine tissues as a template. Thus, the primer design was appropriate for nested PCR analysis.

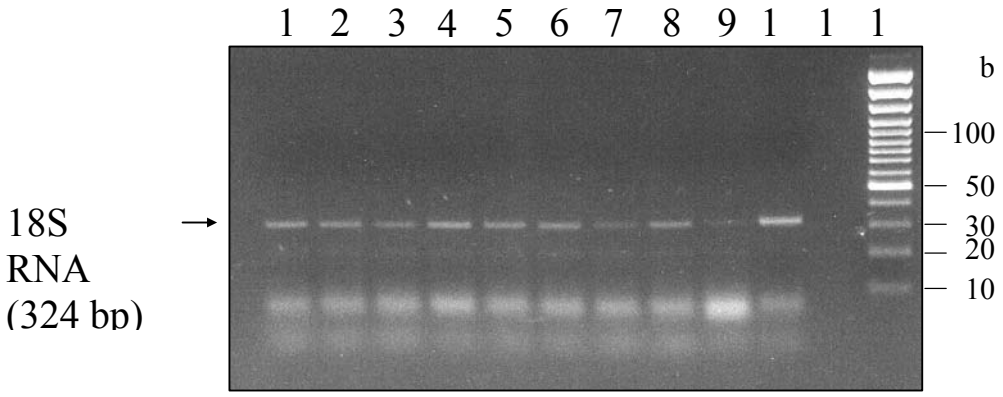

Figure 10: PCR amplification of 18S rRNA.

Total RNA was isolated from paraffin-embedded lymph node tissue derived from dogs either irradiated with 3 mGy/d (lane 1-5, dogs 3495, 3298, 3370, 3062, 3062) or non-irradiated (lane 6-10, dogs 0593, 1031, 1031, 2118, 1026). RNA was reverse transcribed using random hexamer primers and used as template for PCR with 18S rRNA primers. Aliquots of the PCR products (10 µl each) were separated on a 2% agarose gel. A sample containing water instead of cDNA was included as a negative control (lane 11). A 100 bp DNA marker served as a size standard (lane 12).

The lymph node cDNA samples were first used as templates for nested PCR amplification of the house-keeping gene β-actin. After only one round of amplification, no PCR products were detectable on a 2% agarose gel. However, a single band with the expected size of 142 bp was found in each sample after a second round of amplification (Figure 11). Sequencing of the DNA band confirmed the amplification of β-actin. As additional control, RNA not reverse transcribed into cDNA was amplified (not shown). No signals were found indicating that no genomic DNA was present in the RNA preparation and that the PCR products detected above were thus due to the amplification of cDNA. The next aim was to detect mRNA of the adhesion molecules and cytokines of interest. As the optimal annealing temperatures for the respective primer pairs differed from each other, cDNA had to be separately amplified with each primer pair. As shown in Figure 12, ICAM-1 mRNA was amplified from one cDNA sample from lymph node tissue from a non-irradiated control dog. From the same cDNA, also CD44 mRNA was amplified (not shown). Processing of larger numbers of tissue sections was

restricted by the limited amount of canine material provided for this study. Another problem is that nested RT-PCR does not allow a quantitative but only a qualitative conclusion.

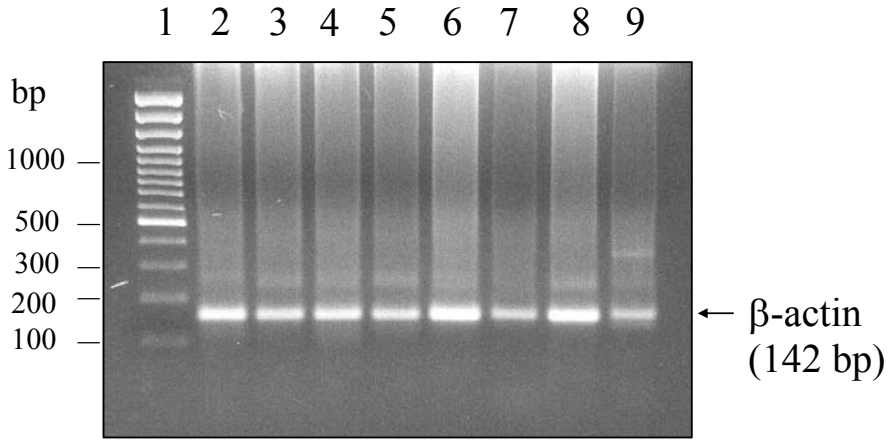

Figure 11: Nested PCR amplification of actin mRNA.

Total RNA was isolated from paraffin-embedded lymph node tissue derived from dogs either irradiated with 3 mGy/d (lane 2-5, dogs 3495, 3298, 3370, 3062) or non-irradiated (lane 6-9, dogs 0593, 1031, 2118, 1026). The RNA was reverse transcribed using random hexamer primers and used as template for PCR with actin specific primers. Aliquots of the PCR products (10 µl each) were separated on a 2% agarose gel. A 100 bp DNA marker served as a size standard (lane 1).

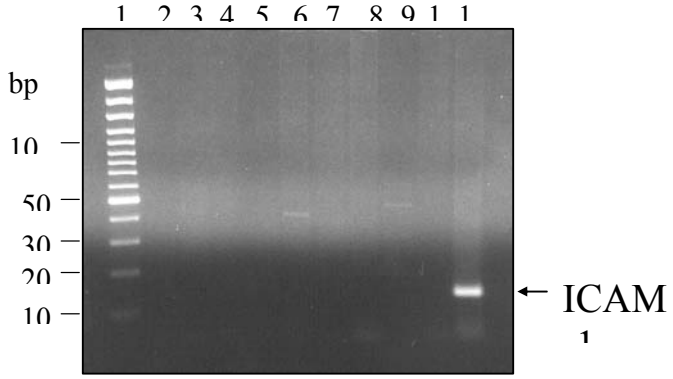

Figure 12: Nested PCR amplification of ICAM-1 mRNA.

Total RNA was isolated from paraffin-embedded lymph node tissue derived from dogs either irradiated with 3 mGy/d (lane 4-7, dogs 3495, 3298, 3370, 3062) or non-irradiated (lane 8-11, dogs 0593, 1031, 2118, 1026). RNA was reverse transcribed using random hexamer primers and used as template for PCR with ICAM-1 specific primers. Aliquots of the PCR products (10 µl each) were separated on a 2% agarose gel. Samples containing water instead of cDNA were included as negative control for the first and second PCR (lane 1, 2). A 100 bp DNA marker served as a size standard (lane 1).

8. Results of RTQ-PCR Analysis

The PPD of the three genes worked in fresh dog material (figure 13). However, in the degraded material only the differential gene expression of ß2-Integrin after 37.5 mGy/d could be calculated. It was in the range of control values (1.3).

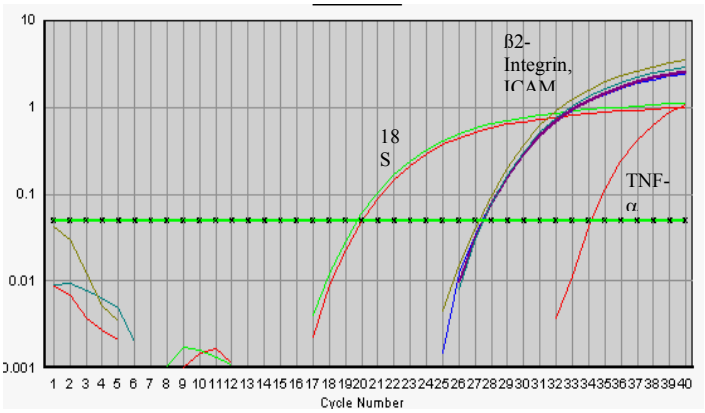

Figure 13: Detection of ß2-Integrin, ICAM and TNF-α mRNA (two repeats each) in fresh tissue from control dogs. For normalization purposes 18 SrRNA was measured.

9. Results of Gene-Array (Non-Radioactive)

The total amount of RNA in the dog material is extremely small (IV.6). For gene-array analysis total RNA was extracted from altogether 16 lymph node tissue blocks, 8 from each group, as described before. Total RNA isolated from each group was combined separately. The GEArray assay normally requires an amount of 5-10 µg of total RNA. Therefore, to increase the amount of input RNA, both total RNA samples were amplified twice using the message amplification antisense RNA-kit. The input RNA requirements for amplification of antisense RNA include RNA quantity, RNA purity and RNA integrity all of which could not be verified due to the limited amount of material. After message amplification, the antisense RNA was ready for labeling via reverse transcription for GEArray. As shown in Fig. 14, hybridization signals for genes related to apoptosis were detected on both membranes. A quantitative analysis of gene expression of both groups normalized to the expression of the housekeeping genes (figure 15) shows a slight but not significant down-regulation of all genes related to apoptosis with the exception of trail and caspase 10, which both are slightly, but again not significantly up-regulated. These preliminary results suggest that there are no significant differences in expression of the genes under study both in the control group and the 3 mGy/d group. If at all, an effect would be a down-regulation of the apoptosis related genes seen in figure 15. This fact certainly has to be proved in a higher number of samples. But already now this observation is in accordance with the results of TdT-staining, where there was also no significant difference regarding the detection of strand breaks within the two groups of 0 and 3 mGy/d. Two separate gene-array experiments with 1 and 3 tissue blocks used for RNA-isolation only yielded two and three positive gene targets.

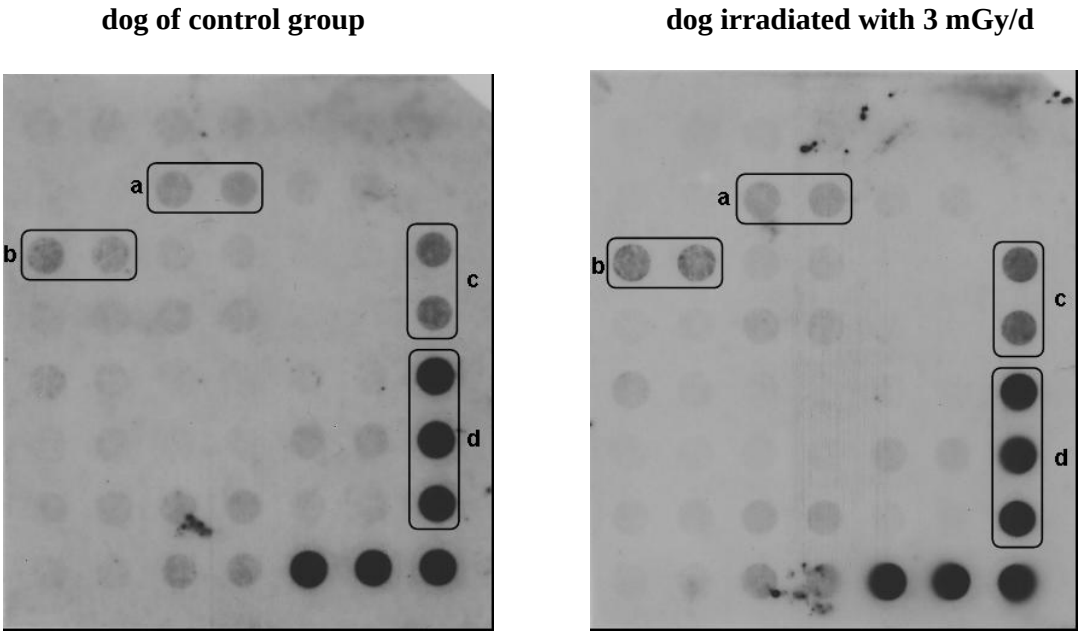

Figure 14: Analysis of differential gene expression using GEArray assay for genes related to apoptosis . Total RNA was isolated from paraffin-embedded lymph node tissue derived from 8 dogs irradiated with 3 mGy/d (3 mGy/d group) and 8 dogs non-irradiated (control group). Biotinylated cDNA probes were synthesized from both total RNA samples by reverse transcription and hybridized to gene-specific cDNA fragments spotted on the membranes. The expression levels can be detected by chemiluminescence signal using alkaline phosphatase substrate. Legend: a = bcl-x; b =caspase10; c =β-actin and d = GAPDH

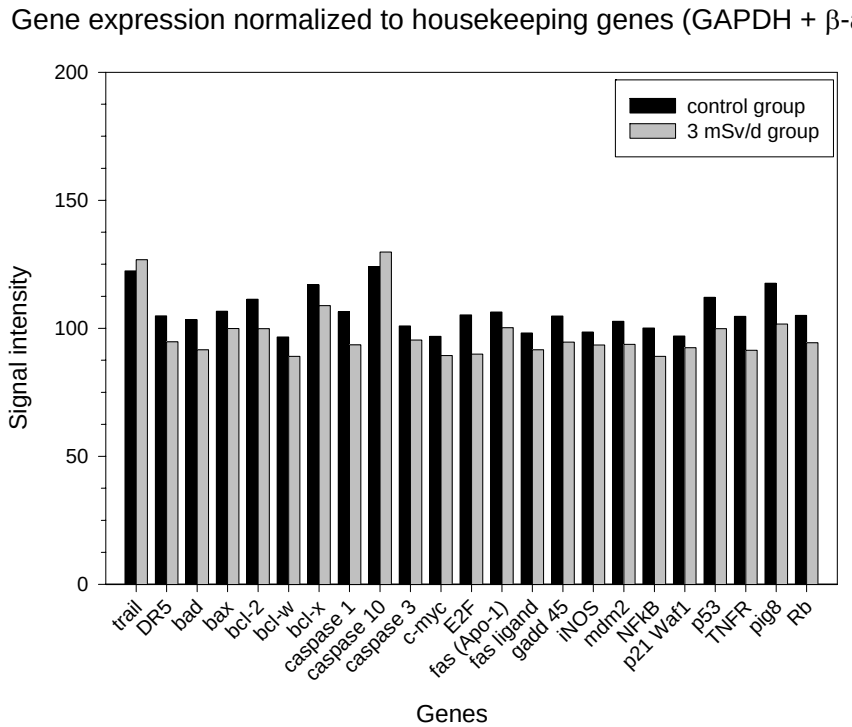

Figure 15: Quantitative analysis of differential gene expression using GEArray assay for genes related to apoptosis (data from figure 14) normalized to housekeeping gene expression.

10. Results of Gene-Array (Radioactive, 12.000 Gene Targets)

Total RNA was extracted from three remaining blocks. But again, only a few expressed genes were found using the array with 12.000 genes. In total not more than 34 genes showed a hybridisation signal (data not shown here). Again, one possible and probable reason is that the amount of RNA obtained from the remaining tissue blocks was insufficient. As compared to the gene array described before the 12 k array requires an at least 3 to 4-fold increased amount of RNA. Unfortunately, there was not enough material left to repeat the experiment.

11. Conclusion of RNA-Analysis Experiments

It could be shown that a set of different methods principally works with archived canine material. A comparison of two different Gene-Array systems showed that this method depends on the amount of RNA inserted. As there is much RNA-degradation in the available canine material, many more tissue blocks have to be used for future studies. A pooling of tissue blocks from the same dose group is recommended (up to 50 – 100) blocks each. Therefore, these experiments will be costly and use much material as they will be time consuming. For this reason future experiments have to be well prepared. Suitable canine gene targets with well known sequences have to be identified and adequate gene-array systems have to be tested with regard to homology to the canine system. In an initial step, RT-PCR methods will be the methods of first choice. The identification of relevant targets should be based on protein analysis (e.g. immunohistochemistry).

12. Results of DNA-Isolation

The successful isolation of DNA could be demonstrated by its amplification using nested PCR (not shown). The same PCR primers and conditions as described above for the analysis of gene expression could be applied. As no information on the intron-exon structures of genes of interest was available, PCR primers could not be chosen for intron-spanning and thus amplify DNA and RNA depending on the experimental settings. Of course, analyzing RNA demanded complete digestion of DNA.

13. Results of Telomer-FISH

The telomer-FISH method worked well with dog skin (figure 16). But only a few sections were used so that presently there is no clear trend of dose-dependent telomer signals.

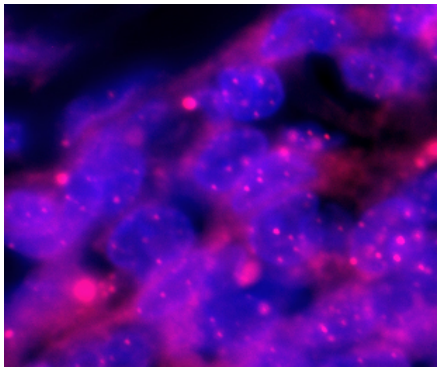

Figure 16: Dog skin irradiated with 18.8 mGy/d

Statistical Design and Methodologies

The basic results of the immunohistological and TdT-staining are images of the histological preparations. To perform fully or semiautomized quantitative evaluation, computer-based approaches for image analysis methods have to be considered. Although this is an area of intensive research in computer science, no generally applicable methods for quantification of immunohistological stainings are established. In the case of immunohistological and TdT images in these studies it seems promising to use (pixel-based) area information as a final outcome which can be extracted by sequences of rather defensive image processing algorithms (Parker 1997, Thompson and Shure 1995). A first test for feasibility of this approach using the embedded dog material showed promising results.

14. Concluding Remarks and Discussion

First results of this feasibility study showed that evaluation of the stored material of the chronically irradiated dogs with modern molecular biological techniques proved to be successful and extremely promising. Therefore an in deep analysis of at least part of the huge amount of remaining material is of outmost interest. The methods applied in this feasibility study were pathological evaluation with different staining methods, protein analysis by means of immunohistochemistry, strand break analysis with the TdT-assay, DNA- and RNA-analysis as well as genomic examination by gene array. Overall more than 50% of the investigated material could be used. Based on the results of this study a new preapplication is attached. In particular the results of an increased stimulation of the immune system within the dogs of the 3mSv group as both compared to the control and higher dose groups gives implications for the in depth study of the cellular events occurring in context with low dose radiation. Based on the findings of this study a further evaluation and statistically analysis of more material can help to identify promising biomarkers for low dose radiation. A systematic evaluation of a correlation of dose rates and strand breaks within the dog tissue might moreover help to explain mechanisms of tolerance to IR. One central problem is that most sequences for dog specific primers are not known yet. The discovery of the dog genome is still under progress. In this study the isolation of RNA within the dog tissue was successful. But up to now there are no gene arrays or gene chips commercially available, tested and adapted for canine tissue. The uncritical use of untested genomic test systems for canine tissue seems to be ineffective at the moment, time consuming and ineffective. Next steps in the investigation of genomic changes after IR within the stored dog tissue should be limited to quantitative RT-PCR of tested primer sequences for the dog. A collaboration with institutions working in the field of the discovery of the dog genome could have synergistic effects.