**Ku70/80 gene expression and DNA-dependent protein kinase (DNA-PK) activity do not correlate with double-strand break (dsb) repair capacity and cellular radiosensitivity in normal human fibroblasts**

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**Summary**
The expression of the Ku70 and Ku80 genes as well as the activity of the DNA-dependent protein kinase (DNA-PK) were studied in 11 normal human fibroblast lines. The proteins studied are known to be part of a double-strand break (dsb) repair complex involved in non-homologous recombination, as was demonstrated for the radiosensitive rodent mutant cell lines of the complementation groups 5–7. The 11 fibroblast lines used in this study represent a typical spectrum of normal human radiosensitivity with the surviving fraction measured for a dose of 3.5 Gy, SF\(_{3.5\,\text{Gy}}\), ranging from 0.03 to 0.28. These differences in cell survival were previously shown to correlate with the number of non-repaired dsbs. We found that the mRNA signal intensities of both Ku70 and Ku80 genes were fairly similar for the 11 cell lines investigated. In addition, the DNA-PK activity determined by the pulldown assay was fairly constant in these fibroblast lines. Despite the correlation between cell survival and dsb repair capacity, there was no correlation between dsb repair capacity and DNA-PK activity in the tested normal human fibroblast lines. Obviously, in this respect, other proteins/pathways appear to be more relevant.

**Keywords:** DNA-dependent protein kinase; Ku70/80; double-strand break repair; radiosensitivity; normal human fibroblasts

In radiotherapy, the maximum dose used to cure a patient from a tumour is partially limited by the occurrence of severe late normal tissue reactions. The extent of these reactions varies individually and shows a broad spectrum even after identical or comparable schemes of irradiation (Bentzen and Overgaard, 1994; Turesson et al, 1996).

There is now increasing evidence that the extent of late normal tissue reactions may depend to a considerable extent on the intrinsic cellular radiosensitivity (Geara et al, 1993; Bentzen and Overgaard, 1994; Ramsay and Birrell, 1995; Burnet et al, 1996; Johansen et al, 1996). So far, the factor(s) determining the intrinsic cellular radiosensitivity are not definitely known. For normal human fibroblasts, radiation-induced loss of cellular reproductivity is known to result from chromosomal damage (Cornforth and Bedford, 1987) and also from a permanent arrest at the G\(_1\)/S border (Di Leonardo et al, 1994). Both of these effects appeared to be determined by non- or misrejoined DNA double-strand breaks (dsbs). This was recently observed in our laboratory for a variety of normal human fibroblasts (Dikomey et al, 1999), where a good correlation between the cellular radiosensitivity and the number of non-repaired double-strand breaks was found.

Up to now, the best known complex involved in mammalian double-strand break repair is the DNA-dependent protein kinase (DNA-PK) complex. This complex consists of the DNA binding subunits Ku70/80 (XRCC6/XRCC5) and the catalytic subunit DNA-PKcs (XRCC7) and is known to be part of the non-homologous recombination pathway, which is considered to be the dominant dsb repair mechanism active in mammalian cells (Jackson and Jeggo, 1995). Additionally, in primate cells, this protein complex interacts with the Ku80-related protein KARP-1 (Myung et al, 1997). In rodent cells, any defect in this complex was found to result in a depressed repair capacity and an enhanced cellular radiosensitivity (Zdzienicka, 1995). However, in human cell lines (mostly human tumour cell lines) no such correlation was found (Allalunis-Turner et al, 1995; Nicolas et al, 1996; Lu et al, 1998). Up to now, there is only one human glioma cell line known in which radiosensitivity clearly correlates with a DNA-PKcs transcriptional defect (Lees-Miller et al, 1995).

This discrepancy between rodent and human data was the basis of the present study, in which we wanted to investigate whether in normal human fibroblasts the cellular radiosensitivity and the extent of double-strand break repair also depended on the DNA-PK complex. The study was performed with 11 normal human fibroblast lines showing a clear difference in dsb repair capacity that correlated with the cellular radiosensitivity (Dikomey et al, 1999). The expression of the Ku70 and the Ku80 gene was determined by Northern blot analysis and the activity of the DNA-PK complex was detected by the pulldown assay (Finnie et al, 1995).

**MATERIALS AND METHODS**

**Cell culture**

The human skin fibroblast lines used were originally established from skin biopsies of women who had received post-mastectomy...
radiotherapy 10–12 years ago (Johansen et al, 1994, 1996). The biopsies were taken from non-irradiated areas. Fibroblasts were grown as a monolayer in Amniomax C100 medium containing 15% supplement and fetal calf serum (7.5% each). CHO-K1 and xrs5 cells were grown in minimal essential medium (MEM), α-modified, containing 5% fetal calf serum (FCS). All cell cultures were incubated at 37°C, 8% carbon dioxide and 100% humidity. All experiments were performed with plateau phase cultures, the human fibroblasts being in passages 6–8.

**Northern blot hybridization**

Total RNA was extracted according to the Fast-Prep System (Dianova, Hamburg, Germany). Total RNA (5 μg) was run in a 1% denaturing agarose gel. Subsequently, RNA was transferred to a nylon membrane (Hybond N+, Amersham, Braunschweig, Germany) and was fixed by UVC cross-linking.

Nylon membranes were prehybridized for 7 h in hybridization buffer containing 50% deionized formamide, 20% 5X buffer [250 mM tris-HCl pH 7.5, 1% bovine serum albumin (BSA) fraction 5, 1% polyvinylpyrrolidone, 0.5% sodium pyrophosphate, 5% sodium dodecyl sulphate (SDS), 1% Ficoll 400], 20% dextran sulphate (50%), 1 M sodium chloride, 0.1 mg ml⁻¹ calf thymus DNA (ultrasound degraded, GibcoBRL, Eggenstein, Germany) at the appropriate hybridization temperature. After addition of the heat-denatured specific DIG-labelled probe, hybridization was carried out overnight at the appropriate temperature (β-actin probe, 42°C; Ku70 probe, 48°C; Ku80 probe, 42°C). After stringency washes (2 × 20 min, 2 × SSC + 0.1% SDS, room temperature; 2 × 15 min, 0.5 × SSC + 0.1% SDS, appropriate temperature, i.e. β-actin probe, 47°C; Ku70 probe, 66°C; and Ku80 probe, 52°C), the hybridization signal was detected via chemiluminescence by using CDP-Star (Boehringer, Mannheim, Germany). Light emission was detected either by exposure of a sensitive film (Hyperfilm ECL, Amersham) or by direct recording and subsequent analysis (Molecular Light Imager – Night OWL; software, winlight; Berthold, Isernhagen, Germany).

Nylon membranes were stripped with Northern probe-stripping solution (50% formamide, 50 mM tris-HCl pH 8, 1% SDS) according to the Boehringer manual (Boehringer).

For the preparation of the specific DNA probes, XL1 Blue bacteria [genotype recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, (F'traD36, proAB, lacIQZAM15, Tn10(tet))] were used, which were transformed with pGem-plasmid containing the human cDNA of either the Ku70 or the Ku80 gene (p70-4z or p80-4z) and which were kindly provided by Dr H Feldmann, Munich, Germany. Bacteria were grown in LB-medium containing 50 μg ml⁻¹ ampicillin. Plasmids were isolated according to the Plasmid Maxi Kit (Qiagen, Hilden, Germany) and were cut with the appropriate restriction enzymes (p70-4z, EcoR1; Pst1; p80-4z, Pst1, Smal; Pharmacia Biotech, Freiburg, Germany). Reaction products were separated in a 1% non-denaturing agarose gel. The resulting DNA fragments representing the specific DNA probes (Ku70, ~1000 bp; Ku80, ~400 bp) were isolated according to the QIAquick Kit (Qiagen). These two probes were DIG-labelled by the random primed labelling method (Boehringer). As specific β-actin probe, a 40-base single-stranded synthetic oligonucleotide (62.5% GC content, Oncogene Science, Dianova, Hamburg, Germany) was used, which was DIG-labelled by the 3’ end-labelling method (Boehringer).

**RESULTS**

**Gene expression of Ku70 and Ku80**

The expression of the two DNA repair genes Ku70 and Ku80 was examined in 11 different normal human fibroblast lines. The cells were grown to confluence and the gene expression was detected via chemiluminescence by using DIG-labelled specific DNA probes. As demonstrated in Figure 1, each of the tested cell lines possesses a Ku70 signal of about 2.2 kb and two variants of Ku80 transcripts of about 2.4 kb and 3.5 kb, respectively, which is in agreement with literature (Cai et al, 1994). As a control for gel loading, the signal intensity of β-actin was used (Figure 1C).
Chemiluminescence detection via exposure of a light-sensitive film. Hybridization with the appropriate DIG-labelled DNA probe and subsequent human fibroblast lines. Total RNA (5 μg) of 11 independent RNA preparations were normalized to the mean value. Error bars represent s.e.m. of three replicates, showing that the expression of these genes is fairly constant for the 11 cell lines. The small differences observed were considered to result from variations in the preparation of the total RNA rather than from biological differences in the level of gene expression because the ranking of gene expression varied between experiments with different RNA preparations (as indicated by the error bars).

DNA-PK activity

The activity of the DNA-PK (which represents a serine/threonine kinase) was determined by the pulldown assay (Finnie et al, 1995).

Figure 1 Gene expression of Ku70, Ku80 and β-actin of 11 different normal human fibroblast lines. Total RNA (5 μg) was analysed by Northern blot, hybridization with the appropriate DIG-labelled DNA probe and subsequent chemiluminescence detection via exposure of a light-sensitive film.

Figure 2 Relative gene expression of Ku70 and Ku80 of 11 different normal human fibroblast lines. Data were obtained from blots like that shown in Figure 1, however the hybridization signals were determined directly via the light emission recorded by a highly sensitive camera. The data were normalized to the β-actin signal to correct for gel loading. The Ku80 value represents the sum of both transcripts. For better comparison, all values were normalized to the mean value. Error bars represent s.e.m. of three independent RNA preparations.

Figure 3 Activity of DNA-PK determined for 11 normal human fibroblast lines. Protein (150 μg) of the appropriate whole cell extract was used. All data were corrected for the background, i.e. c.p.m. in the absence of peptide. The error bars represent s.e.m. of two independent experiments, with triplicate determination.

The specificity of this assay was tested by determination of the [32P] transfer onto a specific peptide compared with the transfer onto a mutated peptide, which lacks a DNA-PK-specific phosphorylation site, or the transfer in the absence of peptide for background evaluation. In the presence of the specific peptide, there was a clear kinase activity. In contrast, using the mutated peptide, the activity detected was similar to the background level (data not shown). These findings clearly demonstrated that the activity detected results from the DNA-PK complex and not from other kinases.

Figure 4 Relation between cellular survival, double-strand break repair capacity and DNA-PK activity in 11 normal human fibroblast lines. (A) Correlation between cell survival and dsb repair capacity. Cell survival after 3.5 Gy was determined for plateau phase cells by colony-forming ability, with delayed plating (14 h) after irradiation; the repair capacity was expressed by the number of non-repaired dsbs detected by constant field gel electrophoresis as a fraction of DNA released from the plug 24 h after irradiation with 100 Gy; data were taken from Dikomey et al (1999). (B) Lack of correlation between dsb repair capacity and DNA-PK activity. The number of non-repaired dsbs were determined as described above; data of DNA-PK activity were taken from Figure 3.
Relation between DNA double-strand break repair capacity and DNA-PK activity

The human fibroblast lines used in the present study were previously shown to differ in their cellular radiosensitivity, with the surviving fraction measured for a dose of 3.5 Gy, SF$_{3.5}$, ranging from 0.03 to 0.28. These variations in radiosensitivity correlate well with the double-strand break repair capacity, as demonstrated by the number of non-repaired double-strand breaks detected 24 h after irradiation with 100 Gy (Figure 4A). However, as demonstrated in Figure 4B, the double-strand break repair capacity of these fibroblast lines does not correlate with the DNA-PK activity. Cell lines with distinct differences in the number of non-repaired double-strand breaks did not show a variation in the DNA-PK activity.

DISCUSSION

The knowledge of proteins defining the cellular radiosensitivity is of profound interest in understanding the clinical phenomenon of different, individual radiosensitivity. In the present study, it was tested for the first time whether the cellular radiosensitivity and double-strand break repair capacity of normal human fibroblasts is determined by the activity of the DNA-dependent protein kinase. This complex is known to participate to a non-homologous recombination pathway, which is the dominant repair mechanism active in mammalian cells (Jackson and Jeggo, 1995).

The experiments were carried out with confluent cultures mainly consisting of G1 cells (>95%) to avoid cell cycle-dependent alterations because the DNA-PK activity is known to vary with the cell cycle with a maximum in G1 and a minimum in the S-phase (Lee et al, 1997). In these 11 cultures, both the level of gene expression tested for Ku70 and Ku80 and the activity of the DNA-PK were found to show only a fairly small variation (Figure 2 and Figure 3). On average, the deviation from the mean was less than 9% for the gene expression and 11% for the DNA-PK activity.

The 11 fibroblast lines used are known to exhibit a typical spectrum of normal radiosensitivity with SF$_{3.5}$, ranging from 0.03 to 0.28 (Dikomey et al, 1999). It was demonstrated here that the variation in cellular radiosensitivity found for human fibroblasts cannot be attributed to a variation in DNA-PK activity. Similar results were previously obtained for ten human tumour cell lines (Allalunis-Turner, 1995) and for a sensitive non-tumour cell line (Lu et al, 1998). Only for the human glioma line MO59J could the increase in cellular radiosensitivity be associated with a defect in the DNA-PK complex (Lees-Miller, et al, 1995).

It was also shown here that in human fibroblasts the DNA-PK activity did not correlate with double-strand break repair capacity (Figure 4B), although these fibroblasts were previously observed to show a broad range in repair capacity which was found to correlate with the respective cellular radiosensitivity (Figure 4A, Dikomey et al, 1999). This result illustrates that in human fibroblasts the variation in double-strand break repair capacity cannot result from differences in the DNA-PK activity. These results are in contrast to data obtained for rodent cell lines of the complementation groups 5–7, for which an increase in radiosensitivity could be ascribed to a decrease in the DNA-PK activity (Weaver, 1995). However, these cell lines cannot be used as a general model of the impact of the DNA-PK complex because the reduction of DNA-PK activity was always caused by a genetic defect and not by a variation in the transcription or translation level. Probably, an activity of the DNA-PK other than the kinase activity is relevant for double-strand break repair, but this activity then has to be independent from the kinase activity.

In this context, it is also important to note that in normal human fibroblasts the activity of DNA-PK was about 70 times higher than in normal rodent cells (data not shown; Anderson and Lees-Miller, 1992). This difference, however, did not result in an enhanced repair capacity of human cells (Dikomey et al, 1998). This observation indicates that the DNA-PK complex is not rate limiting for dsb repair. Probably, other enzymes that are less expressed compared with the DNA-PK are rate limiting and, therefore, more relevant for double-strand break repair. From the eight genes identified so far to affect the cellular radiosensitivity of mammalian cells (XRCC1–XRCC8), the three genes XRCC5–XRCC7 can be excluded from determining the double-strand break repair capacity of human fibroblast lines because of the data shown here. Also, the XRCC1 and the XRCC3 genes can be excluded because the products of these genes are known to participate in single- but not in double-strand break repair (Zdzienicka, 1995). In this respect, the XRCC8 gene is also not relevant, because cells mutated in this gene do not show normal single-strand break and double-strand break repair (Zdzienicka, 1995). The XRCC2 gene product can also be rejected because a defect in this gene was found only to affect the fidelity but not the capacity of double-strand break repair (Zdzienicka, 1995). Probably, the XRCC4 gene is of relevance. It was recently shown that the corresponding gene product is a stimulating factor of DNA ligase IV, acting downstream of the Ku70/Ku80 proteins and thereby representing part of the DNA-PK pathway (Critchlow et al, 1997; Grawunder et al, 1997; Wilson et al, 1997). Also the ATM gene might be of importance because a defect in this gene was found to result in a reduced double-strand break repair capacity (Dikomey et al, 1998; Blöcher et al, 1991).

It should be kept in mind that cell lines defective in the DNA-PK pathway are still able to repair more than 50% of the double-strand breaks induced (Kemp et al, 1994; Hendrickson et al, 1991). These double-strand breaks are repaired either by another non-homologous recombination pathway or even by homologous recombination, and it remains to be shown whether cellular radiosensitivity is mainly determined by these mechanisms.

In conclusion, the data presented demonstrate that in normal human fibroblast lines double-strand break repair capacity and cellular radiosensitivity do not correlate with the DNA-PK activity. Obviously, in this respect other proteins or pathways are more relevant.

ACKNOWLEDGEMENTS

The authors thank Dr Heidi Feldmann (Institute of Biochemistry, University of Munich) for the kindly gift of the Ku70/Ku80 transfected bacteria XL1 Blue and Dr Jose Coco Martin (The Netherlands Cancer Institute, Amsterdam) for the mutated peptide. We also thank Professor Dr Armin Hildebrandt (UFT, University of Bremen) for the feasibility to use the highly sensitive camera system and especially Andreas Marquardt and Manfred Pisula for their practical help. In addition, we are grateful to Kerstin Borgmann, Dr Jochen Dahm-Daphi and Dr Ingo Brammer for valuable discussions.

This work was supported by the Deutsche Forschungsgemeinschaft, grant no. Di 457/2–1 (ED), and by the Danish Cancer Society, grant no. 9510054 (JO), and by the BIOMED project no. PL950638.
ABBREVIATIONS
DIG, Digoxigenin; DNA-PK, DNA-dependent protein kinase; dsb, double-strand break; dsDNA, double-stranded DNA; SF, surviving fraction; XRCC, X-ray cross complementing.

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British Journal of Cancer (1999) 79(7/8), 1037–1041