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Enhanced DNA damage-induced p53 peptide phosphorylation and cell-cycle arrest in Sjögren's syndrome cells

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Abstract

Background Cells from primary Sjögren's syndrome (SS) patients have been reported to show alterations in DNA repair and p53 expression. The DNA-dependent protein kinase (DNA-PK) autoantigen may be involved in both of these alterations in relation to cellular DNA damage responses. We conducted this study of cell-cycle kinetics and p53 to find additional evidence for an abnormal stress response role in the pathogenesis of SS.

Design DNA-dependent protein kinase activity, p53 peptide phosphorylation and p53 protein levels were determined in gamma-irradiated long-term T lymphocyte cultures. Cell-cycle progression of peripheral blood mononuclear cells was analysed with flow cytometry.

Results No significant differences in the DNA-PK activities or p53 protein levels appeared between the SS patients and the healthy individuals. However, patients with the SS hallmark Ro/SS-A and La/SS-B autoantibodies showed enhancement of both p53 peptide phosphorylation ($P = 0.036$) and G1 cell-cycle arrest ($P = 0.015$) in response to gamma radiation.

Conclusions Sjögren's syndrome cells express an enhanced G1 checkpoint function which may be mediated partly by p53 phosphorylation, suggesting that an abnormal stress response in SS is of relevance for the development of this autoimmune disease.

Keywords Autoimmunity, DNA damage response, DNA-dependent protein kinase, Sjögren's syndrome.

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Introduction

Primary Sjögren's syndrome (SS) is a chronic, systemic autoimmune disease with diminished exocrine glandular function, focal lymphocytic infiltration, and the characteristic symptoms of dry mouth and irritable eyes. However, a number of other organ systems may also be affected, leading to a broad spectrum of both exocrine and nonexocrine manifestations (reviewed in [1–3]). The aetiology of the disease is unknown but is often considered to be an interaction

between constitutional/genetic and environmental factors leading to autoimmunity [4].

A characteristic property of SS and other systemic autoimmune conditions is the production of autoantibodies directed to intracellular antigens. A number of these antigens are nuclear and are involved in nucleic acid modifications, such as the Ro/SS-A and La/SS-B RNA processing proteins and V(D)J recombinase components, including the DNA-dependent protein kinase (DNA-PK) [5–7]. It has been hypothesized that this autoantibody production is generated during the response to modified self-antigens [4,6,8]. Fox *et al.* [9] have presented a hypothetical model for the aetiology of SS, which includes defects in the rearrangement of lymphocyte antigen receptor genes. This model fits well with the observation that many of the autoantigens in systemic autoimmune diseases are involved in DNA modification. Also in accordance with this model are reports of DNA repair alterations and a reduced frequency of illegitimate V(D)J recombinations in SS lymphocytes [10–13].

DNA-dependent protein kinase, the Ku-component that has been described as an autoantigen in some patients with SS, is important for both DNA double-strand break repair

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and V(D)J recombination [14,15]. DNA-dependent protein kinase is a nuclear serine/threonine protein kinase that is activated upon association with DNA, and is composed of a catalytic subunit, termed DNA-PKcs, and a DNA-targeting component, the heterodimeric Ku protein. The available data lead to a model in which Ku binds to free DNA ends and subsequently recruits DNA-PKcs to DNA, triggering the kinase function of DNA-PK (reviewed in [15]). An efficient target for DNA-PK, as documented *in vitro*, is the p53 protein, with key roles for G1 cell-cycle arrest and apoptosis in response to DNA damage [15–18].

In previous studies of primary SS we have found alterations in DNA repair and an aberrant pattern of stress-specific DNA-binding proteins in SS lymphocytes [10,11,19]. These observations have led to a hypothesis of an altered stress response as a pathogenetic mechanism in SS. To test this hypothesis we have analysed the response to DNA damage of SS lymphocytes, concerning DNA-PK activity, phosphorylation of a p53 peptide, and cell-cycle progression.

Materials and methods

Patients and cells

Peripheral venous blood was obtained from patients with primary SS as well as from healthy individuals. All patients fulfilled both the European classification and the Copenhagen's classification for SS criteria [20,21]. The presence of anti-Ro/SS-A and anti-La/SS-B autoantibodies was determined by immunodiffusion (Immunoconcept, Sacramento, CA). Immediately after the blood was drawn, the mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). T-lymphocyte cultures were established by stimulating the peripheral blood mononuclear cells (PBMC) with $1 \mu\text{g mL}^{-1}$ phytohaemagglutinine (Wellcome Research Laboratories, Beckenham, UK) and then culturing them in $10 \mu\text{g mL}^{-1}$ IL-2 (Roche, Mannheim, Germany) enriched growth medium for 15–21 days. Flow cytometric characterization at the end of the culture time, using monoclonal antibodies, demonstrated that they contained more than 99% CD3+ T lymphocytes and less than 1% CD19+ (B lymphocytes) or CD14+ (monocytes) cells. HeLa and HEp2 cells were from the American Type Culture Collection (Rockville, MD; 2-CCL and 23-CCL, respectively). All cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 25 mM Hepes and $12 \mu\text{g mL}^{-1}$ gentamicin at 37°C in a humidified 5% CO_2 atmosphere.

Ionizing irradiation

Gamma radiation was delivered by a neutron accelerator (Philips, Germany) at a dose rate of 0.70 Gy min^{-1} , at a distance of 50 cm from the cells, which were kept in their

plastic culture flask in complete medium and at room temperature. All cell cultures in the different assays were handled identically with the only difference being exposure to ionizing radiation. Following irradiation, cells were returned to the incubator and harvested at the indicated points of time.

Protein extraction

Protein extracts were prepared by a modification of Dignam *et al.*'s method [22]. Cells were resuspended in a low salt buffer (10 mM Hepes pH 7.6, 25 mM KCl, 10 mM NaCl, 1 mM MgCl_2 , 0.1 mM EDTA, 1 mM DTT, 10% (v/v) mixture of proteinase inhibitors [Complete, Roche]), and incubated on ice. Sedimented nuclei were resuspended in a high salt buffer (10 mM Hepes pH 7.6, 25 mM KCl, 0.4 M NaCl, 1 mM MgCl_2 , 0.1 mM EDTA, 1 mM DTT, 10% Complete) and centrifuged to obtain the nuclear extract. The protein concentration was determined spectrophotometrically using BCA protein assay reagents (Pierce, Rockford, IL).

Analysis of DNA-dependent protein kinase activity

The phosphorylating function of DNA-PK was measured using a synthetic p53 peptide (Promega, Madison, WI) containing a DNA-PK specific phosphorylation site as substrate, including the wild-type p53 amino acids 11 (Glu) – 24 (Lys) but excepting 18 (Thr) and 20 (Ser), which were changed to alanine, leaving serine 15 as a phosphorylation target for DNA-PK [23], as described previously [24]. Briefly, nuclear protein extract was mixed with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the synthetic p53 peptide, with or without sonicated DNA. The DNA-PK activity was calculated as the c.p.m. in the presence of DNA minus the c.p.m. in the absence of DNA. All nuclear extracts were analysed in triplicate, and the variation for each triplet was considered small with an intra-assay coefficient of variation (CV, i.e. SD/mean value) of 8.7%. The interassay CV was estimated using HeLa cells harvested and frozen on the same occasion. Seven determinations, made on seven separate dates, showed a CV of 19.4%.

Flow cytometry

A Coulter Epics XL-MCL flow cytometer (Coulter Corp., Miami, FL) was used. Determination of T- and B-lymphocyte concentrations was performed using monoclonal antibodies (anti-CD3, -CD5, and -CD19; Coulter Corp.) and a fluorescent microsphere reagent (Flow-Count Fluorospheres, Coulter Corp.) as previously described [25]. For cell-cycle analysis a standard total DNA labelling protocol was used, incubating the cells in a hypotonic 0.01 M NaCl, 0.1% NP-40, $50 \mu\text{g mL}^{-1}$ propidium iodide and $10 \mu\text{g mL}^{-1}$ RNase A solution on ice for 10–60 min. At least 8000 events were scored. MacCycle II software (Phoenix Flow, Phoenix, AZ) was used to calculate the fractions of cells located in the different cell-cycle phases.

Western blot analysis

Standard immunoblotting was performed, as described [24]. Membranes were probed with monoclonal mouse anti-p53 antibody (specific for amino acids 46–55 of human p53; clone PAb 1801, Calbiochem, Darmstadt, Germany), and the immunoblots were developed using enhanced chemiluminescence method (ECL; Amersham Pharmacia Biotech, Uppsala, Sweden).

Statistical analysis

The Wilcoxon two-sample test was used for statistical analysis of the data [26]. *P*-values of less than 0.05 were considered statistically significant.

Results

Kinase activity in long-term cultures of T lymphocytes

Long-term cultures of T lymphocytes were established from 10 patients with primary SS (mean age 57 years, range 40–68, nine females and one male; six being positive and four negative for SS-A/SS-B autoantibodies) and five healthy individuals (mean age 53 years, range 48–60, all females). There was no apparent difference between the control and Sjögren groups in cell number doubling time or other growth characteristics. After 15–21 days of cultivation (when a high enough number of cells was reached to make the analysis possible) three culture flasks were exposed to 5 Gy gamma radiation, while another three were left unirradiated. Finally, at 1, 4, and 24 h following irradiation, crude nuclear protein extracts were prepared.

To examine the activity of DNA-PK we assayed the incorporation of ³³P-labelled ATP into a synthetic p53 peptide, containing a DNA-PK specific phosphorylation site, in the presence of sheared DNA. Assays take advantage of the fact that DNA-PK appears to be the major DNA-activated kinase in mammalian cells [27]. Thus, DNA-PK activity is calculated by subtracting the amount of radioactivity incorporated on the p53 peptide in the absence of DNA from the amount of radioactivity incorporated in the presence of DNA.

There was no apparent influence by postirradiation incubation time on the result, with a large interindividual time-point variation showing maximum kinase activity. Therefore, for a comparison between controls and SS patients, the single maximum DNA-PK activity value for each individual (obtained at either 1, 4, or 24 h) was used (Fig. 1a,b) [28]. Although the median values of this DNA-PK activity in both unirradiated and irradiated T lymphocytes was higher in the SS-A/SS-B positive SS subgroup compared with the control group and the SS-A/SS-B negative SS patients, no statistical significance was reached (*P* > 0.05).

The maximum c.p.m. value of each individual for the total phosphorylation of the p53 peptide (determined as the

amount of radioactivity incorporated on the p53 peptide in the presence of DNA without subtracting the amount of radioactivity incorporated in the absence of DNA) is shown in Fig. 1(c,d). No statistically significant difference in the extent of p53 phosphorylation by unirradiated T lymphocytes was found between the controls and the SS individuals (*P* > 0.05) (Fig. 1c), whereas with cells exposed to gamma radiation a significantly stronger reaction was seen among the SS-A/SS-B-positive SS patients (*P* = 0.036), with medians of 60 334 c.p.m. in this SS subgroup and 35 445 c.p.m. in the controls (Fig. 1d).

Western blot analysis of p53 levels

Under normal cell growth conditions p53 is present in very low amounts because of its extremely short protein half-life, which is required for it to stabilize in response to DNA damage [17]. In unirradiated T lymphocytes there was no detectable p53, but after 5 Gy gamma irradiation the p53 level was strongly increased in both SS patients and healthy controls. A total of six SS-A/SS-B-positive SS patients and five controls were analysed. A representative blot including material from three controls and five patients is shown in Fig. 2 (a,b). There was considerable interindividual variation, thereby providing no evidence for a difference in the irradiation-induced amount of p53 between the SS and control groups. A serial dilution of the nuclear protein extract of two individuals presenting with similar p53 band intensities (C2 and S3 in Fig. 2b) expressed the semiquantitative capacity of the immunoblotting procedure, which spanned over at least two 10-folds of protein concentration (Fig. 2c).

Cell-cycle phase distribution

It is well known that p53 activation is associated with G1 cell-cycle arrest [16], and we explored the effects of gamma radiation on cell-cycle progression in PBMC isolated from 20 SS patients (mean age 60 years, range 36–78, 18 females and two males: seven being positive and 13 negative for SS-A/SS-B autoantibodies) and six healthy individuals (mean age 44 years, range 35–54, five females and one male). The PBMC cultures were stimulated for 24 h with PHA before gamma irradiation. At days 0, 2, and 5, samples were drawn from the cultures and analysed for total DNA content by flow cytometry. At day 0 less than 2% of the PBMC had entered the cell cycle, and even at day 2 the fraction of cells in the S and G2 phases was relatively small (< 15%). However, at day 5 more than 40% of the PBMC were in the S and G2 phases, showing that a large proportion of the cells had started cycling, and more than 90% of the cells were found to be CD3+ T cells.

It became clear that exposure to gamma radiation leads to an increased fraction of cells in the G1 phase (Fig. 3). The percentages (median value) of PBMC from healthy individuals located in the G1 phase at 0, 2, and 7 Gy were 56.0 (range 47–64), 53.5 (39–66), and 64.5 (53–74), respectively; in SS-A/SS-B-positive SS patients the corresponding

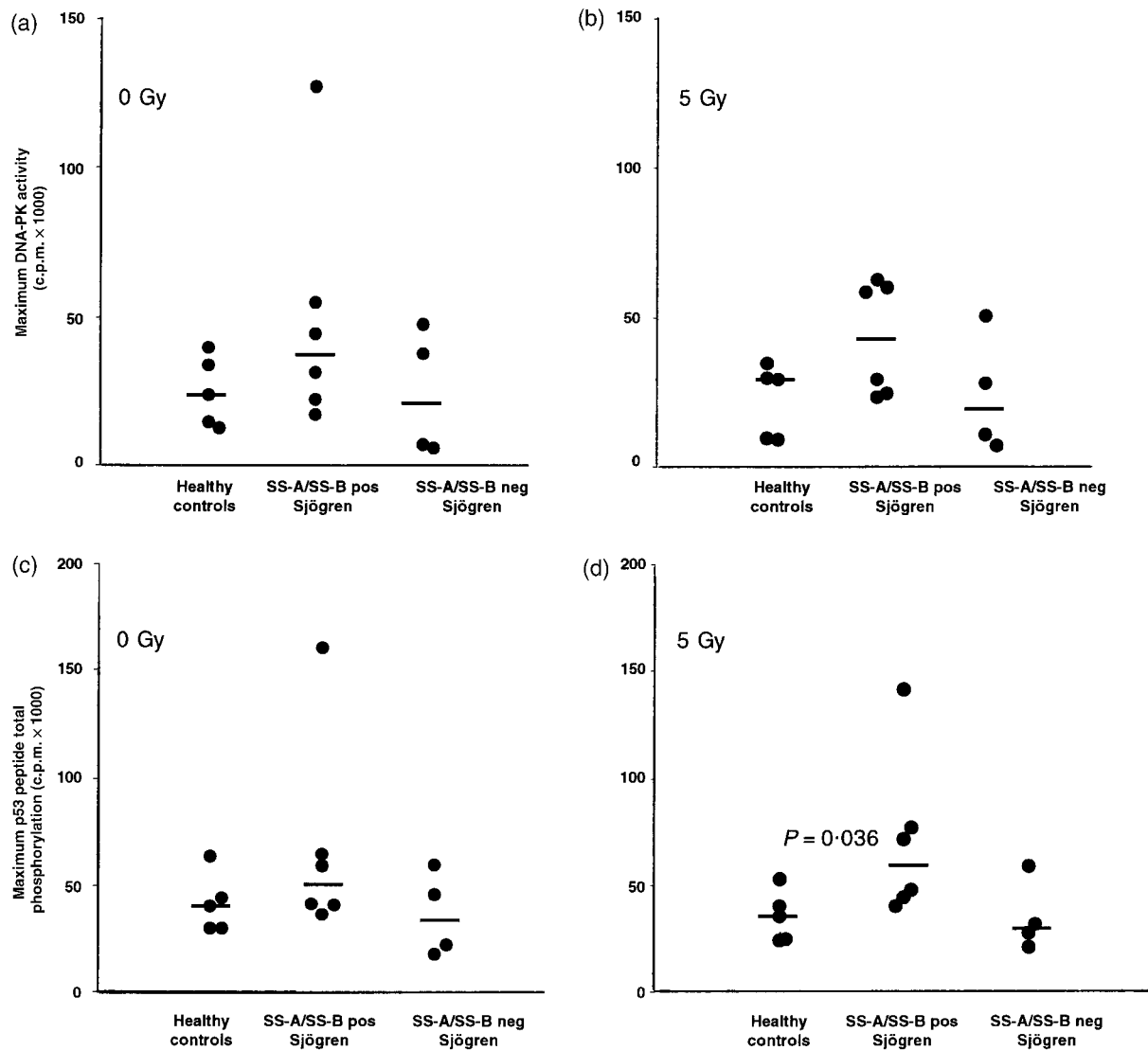


Figure 1 (a,b) DNA-dependent protein kinase (DNA-PK) activity (c,d) and p53 peptide total phosphorylation in T lymphocytes exposed to 5 Gy of gamma radiation, or un-irradiated, and then incubated for 1–24 h. Each dot of the scatter plot represents an individual's maximum value. (a,b) Nuclear extracts derived from long-term cultures of T lymphocytes isolated from healthy controls ($n = 5$), SS-A/SS-B-positive ($n = 6$) and SS-A/SS-

B-negative ($n = 4$) SS patients were analysed for DNA-dependent ^{32}P -incorporation on a synthetic p53 peptide. (c,d) The total phosphorylating activity of DNA-PK as well as other kinases that target the p53 peptide was also analysed. The P -value (d) indicates the enhanced p53 peptide total phosphorylation by SS-A/SS-B-positive SS patients as compared with healthy controls. The horizontal lines show the median values.

figures were 58.0 (36–79), 64.0 (44–86), and 80.0 (56–95); and in the SS-A/SS-B-negative SS patients: 61.0 (37–84), 62.0 (39–82) and 78.0 (51–93). The degree of G1 arrest after DNA-damaging events (i.e. the difference between irradiated and unirradiated PBMC in the fraction of cells located in the G1 cell-cycle phase illustrates the cellular response to DNA damage [29]) was used to compare the SS patients and healthy controls. Healthy individuals showed no G1 arrest at 2 Gy, whereas a G1 increase of 9.5% (median) was found at 7 Gy (Fig. 4). Among the SS-A/SS-B-positive SS patients there was a significantly stronger G1 arrest: 6.0% at 2 Gy and 18.0% at 7 Gy (medians; $P = 0.015$ and 0.032, respectively). There was no statisti-

cally significant difference between the SS-A/SS-B-negative patients and the healthy controls. However, this SS subgroup displayed a large heterogeneity, which could be seen clearly at the 2 Gy dose level. Here, eight patients showed lower values similar to those of the control group, whereas the remaining five patients presented higher G1 arrest values, similar to the SS-A/SS-B-positive subgroup (Fig. 4a).

Effects of gamma radiation on cell numbers

At day 5 after PBMC culture start flow cytometric determination of the T and B lymphocyte numbers was made,

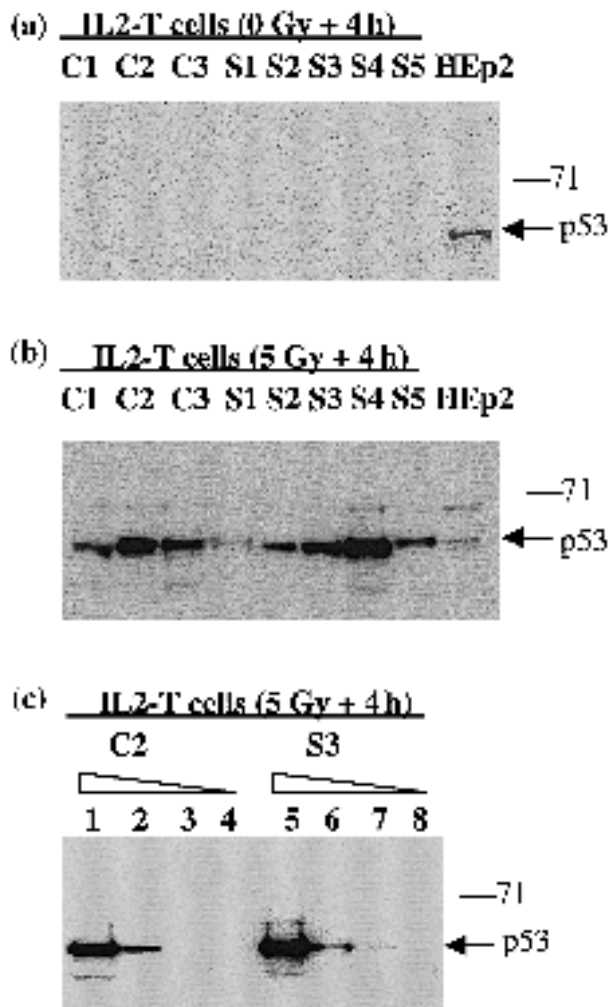


Figure 2 Western blot determination of p53 protein levels in gamma-irradiated T lymphocytes. Nuclear protein extracts from long-term T-cell cultures were separated by SDS-PAGE and immunoblotted with a monoclonal antibody to human p53. The T cells were from three healthy controls (C1–3) and five SS-A/SS-B-positive SS patients (S1–5). HEp2 is a human epithelial tumour cell line, serving as a positive control. (a) T cells with no gamma radiation; (b) T cells exposed to 5 Gy and then postincubated for 4 h; (c) T-cell nuclear protein from individuals C2 and S3 was serially diluted; 10 µg protein in lanes 1 and 5, 3 µg in lanes 2 and 6, 1 µg in lanes 3 and 7, and 0.3 µg in lanes 4 and 8.

including 16 of the SS patients from the cell-cycle study (five being positive and 11 negative for SS-A/SS-B auto-antibodies) and eight healthy individuals (the number of healthy controls was extended after completion of the cell-cycle study). Exposure to 2 and 7 Gy caused a dose-dependent decrease in lymphocyte proliferation with a large inter-individual variation in cell numbers (e.g. at day 5 the range was $67\text{--}450 \times 10^3$ PBMC mL^{-1} for unirradiated cells, with no apparent difference between the control and Sjögren groups). The 2Gy/0Gy cell number quotient (median value) was 0.65 for the healthy controls, 0.40 for the SS-A/SS-B-positive SS patients, and 0.50 for the SS-A/SS-B-negative

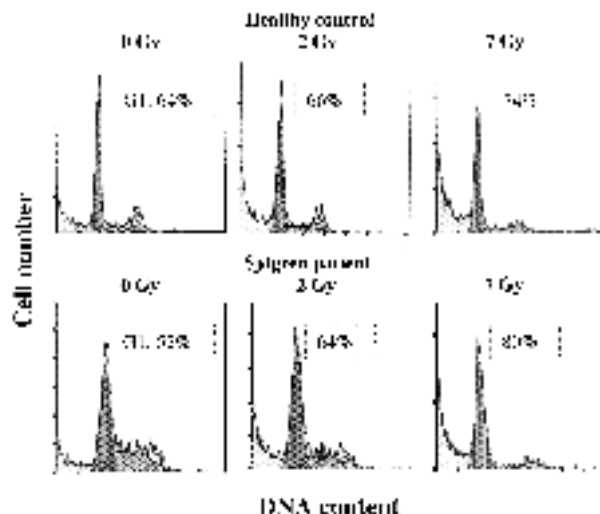


Figure 3 Gamma radiation-induced accumulation of lymphocytes in the G1 cell-cycle phase. Representative experiments are shown, with peripheral blood mononuclear cells from a healthy control and one SS-A/SS-B-positive SS patient that were mitogen-stimulated, exposed to 2 or 7 Gy of gamma radiation 24 h later, and after a further 4 days were stained with propidium iodide and analysed by flow cytometry for total DNA content. Histograms from the MacCycle software are displayed, referring the cells to cell-cycle phases. The resulting value of percentage cells located in G1 is inserted into each histogram.

SS subgroup. The cell concentration in irradiated cultures was divided by that of the corresponding unirradiated samples. The corresponding 7Gy/0Gy quotients (median value) were 0.20, 0.10 and 0.20, respectively. These results cannot be interpreted to indicate an increased radiosensitivity in the SS lymphocytes because of the large interindividual variation, e.g. at 7 Gy the healthy control range was 0.20–0.50 and for the SS patients 0.10–0.60 ($P > 0.05$).

Discussion

DNA-damaging events result in a number of cellular responses, including the temporary halt of cell-cycle progression to allow for DNA repair, and the elimination of severely damaged cells through apoptosis. The tumour suppressor protein p53 plays a central role in these processes [18] and has been identified *in vitro* as a target for the DNA damage-activated DNA-PK, being engaged in both DNA repair and V(D)J recombination [15]. In previous studies of primary SS we have found alterations in DNA repair and an aberrant pattern of DNA damage-inducible proteins in SS lymphocytes [10,11,19]. We therefore considered it interesting to follow up our previous findings with a study of DNA-PK activity in SS lymphocytes.

Our results provided no evidence of abnormal DNA-PK activity in T lymphocytes from SS patients. However, radiation-induced DNA damage resulted in an enhanced total (i.e. the sum of DNA dependent and DNA independent)

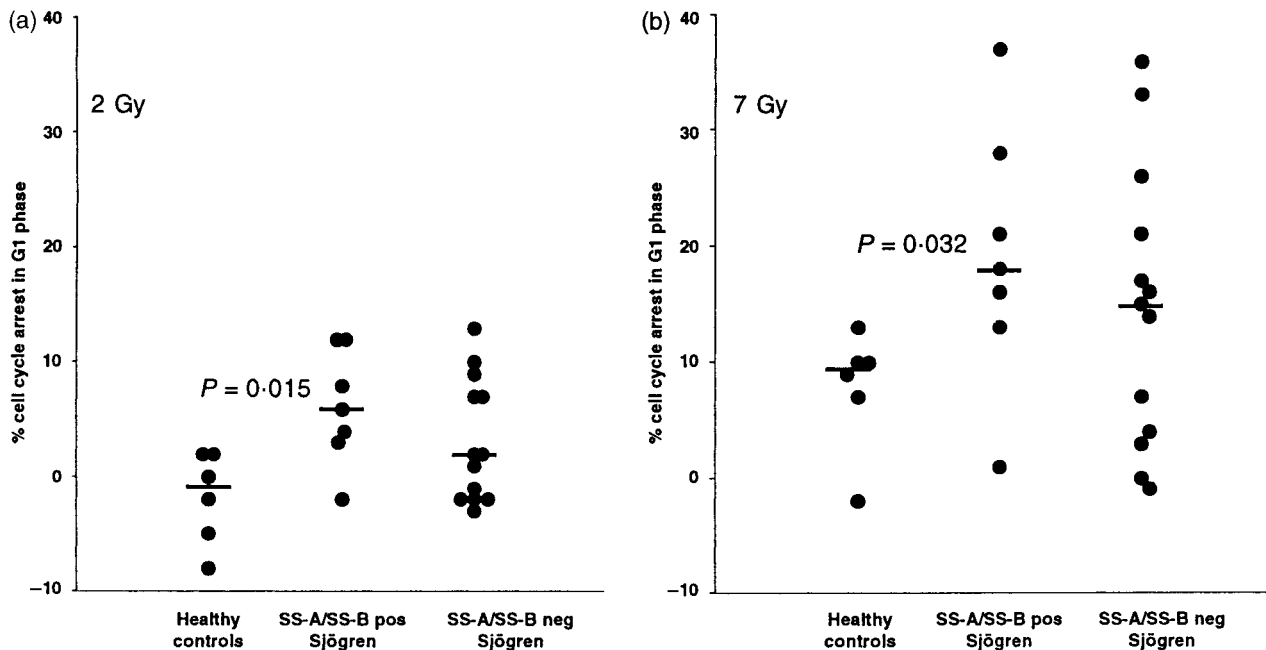


Figure 4 G1 arrest in peripheral blood mononuclear cells from healthy controls ($n = 6$), SS-A/SS-B-positive ($n = 7$) and SS-A/SS-B-negative ($n = 13$) SS patients. The cells were exposed to (a) 2 Gy (b) and 7 Gy of gamma radiation 24 h after mitogen stimulation, and cell-cycle analysis was performed by flow cytometry

determination of total DNA content after a further 4 days of cultivation. The P -values indicate the significantly enhanced radiation-induced cell-cycle arrest by SS-A/SS-B-positive SS patients, as compared with healthy controls. The horizontal lines show the median values.

phosphorylation of the p53 synthetic peptide in the SS-A/SS-B-positive SS subgroup. Initially, the presently used p53 peptide was assumed to be targeted only by DNA-PK [23]. However, in recent years it has been shown that the DNA-PK-related phosphatidylinositol 3-kinase-like proteins ATM (ataxia-telangiectasia mutated) and ATR (ATM-related) are also capable of phosphorylating p53 on serine 15 *in vitro*, i.e. the same amino acid that is phosphorylated by DNA-PK [15,30–34] (being part of the p53 peptide used by us). Ataxia-telangiectasia mutated and ATR have important functions in the signalling of DNA damage [31,34]. It is also possible that signalling of DNA damage by other, as yet unidentified, protein kinases leads to phosphorylation of the used p53 peptide. Accordingly, the significantly higher capacity of T lymphocytes from SS-A/SS-B-positive SS patients to phosphorylate the p53 peptide in response to DNA damage, compared with T lymphocytes from healthy individuals, may be a sum effect of a number of kinases; possibly including DNA-PK.

As a consequence of the enhanced DNA damage-induced p53 peptide phosphorylation, one might expect an elevated level of stabilized p53. However, the p53 level varied considerably between individuals in both the SS group and the control group, and we could not document any difference in the amount of p53 between the two groups. In order to make such a comparison more sensitive, a study of the kinetics of p53 induction during a postirradiation recovery period may be necessary, as well as the inclusion of larger groups of SS patients and healthy controls. Furthermore, it should be noted that factors other than p53 protein levels may

mediate the SS-specific cellular response, e.g. phosphorylation of p53 is known to lead to conformational changes which may affect the ability of p53 to bind to other proteins and to DNA, and thereby increase the activity of p53 [35,36].

G1 phase cell-cycle arrest is one of numerous adaptive and protective activities that are expected to result from the activation of the p53 protein by ionizing radiation [16–18,37]. Moreover, it is known that phosphorylation of p53 on serine 15 in response to ionizing radiation is reduced in A-T cells and that these cells display a diminished radiation-induced arrest in the G1 phase of the cell cycle [32]. One may therefore speculate that our finding of a significantly enhanced G1 arrest in response to gamma radiation in PBMC from SS patients is causally related to the parallel observation of enhanced p53 peptide phosphorylation. However, there is a wide gap between the ability of T-cell lysates to phosphorylate a synthetic peptide *in vitro* and actual phosphorylation of p53 *in vivo*, and, furthermore, the effect of such phosphorylation on the p53 transcription factor function.

In a previous study, Zeher *et al.* [38] found that the increased susceptibility to apoptosis of peripheral CD4+ T cells from SS patients correlates with lymphocyte activation. Perhaps our present finding of an aberrant lymphocyte response to radiation-induced DNA damage is also the result of the immune system's chronic activation occurring in SS? The fact that the analysis of p53 peptide phosphorylation was performed with long-term cultures of T lymphocytes argues against this theory, as it can be deemed

unlikely that an *in-vivo* activation of the SS patients' T lymphocytes would persist for 15–21 days of *in-vitro* cultivation.

Tapinos *et al.* [39] recently reported an overexpression of wild-type p53 in the minor salivary gland biopsy specimens from patients with SS, and suggested a probable role for DNA-damage response genes in the pathogenesis of SS. This report is in accordance with our previous and present observations of an aberrant DNA-damage response in lymphocytes from SS patients. It would be interesting to analyse lymphocytes invading the salivary glands in SS patients with regard to DNA-PK activity and p53-peptide phosphorylation. Unfortunately, the number of lymphocytes in a minor salivary gland biopsy is not significant enough to make an analysis possible using the present method.

To the best of our knowledge, our study is the first that, in a systemic autoimmune disease, shows an enhanced DNA damage response that effects cell-cycle regulation. Further studies are needed to clarify whether or not this effect is specific for SS or may also be found in other systemic autoimmune diseases, as indicated by the overexpression of p53 as well as by somatic mutations in the p53 gene in rheumatoid arthritis [40,41].

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