Primary Sjögren’s syndrome (SS) is characterized by diminished exocrine glandular function, autoantibody production and focal infiltration of B and T lymphocytes. Besides the cardinal symptoms of dry eyes and dry mouth, the disease may be complicated by systemic manifestations from the lungs, kidneys and nervous system, for example, as well as lymphoma [1, 2]. As for many other autoimmune diseases, the aetiology is unknown, but it is considered to be due to interaction between genetic and environmental factors [3]. The genetic factor is often thought to result in alterations in the immune system. However, a recent study showed that genes with a distinct expression pattern in autoimmunity are not necessarily immune response genes, but instead encode proteins involved in apoptosis, cell cycle progression, cell differentiation and cell migration [4].

Human cells can elicit a large set of defence functions for use in stress situations threatening cellular homeostasis, including DNA repair, cell cycle alterations and apoptosis [5, 6]. In previous studies of primary SS we have found alterations in such stress functions in SS lymphocytes; concerning DNA repair, DNA-binding proteins [7–9] and cell cycle arrest in T cells [10]. This has led to the hypothesis of an enhanced stress response as a pathogenetic mechanism in SS [11]. A major cellular component counteracting damage caused by gamma radiation and oxidative stress is DNA-dependent protein kinase (DNA-PK), composed of a catalytic subunit (DNA-PKcs) and the DNA end-binding heterodimeric Ku protein. Ku binds to double-stranded DNA breaks, leading to recruitment of DNA-PKcs to DNA, and activation of its kinase function. This initiates a cascade of events, including DNA-PK-mediated non-homologous end-joining of DNA strand breaks and, most importantly, kinase-dependent accumulation of p53 [12], modulating both cell cycle arrest in order to allow time for the cell to repair damage, and apoptosis eliminating severely damaged cells [13].

Interestingly, the DNA-PK complex, besides its role in DNA repair, forms an essential part of V(D)J recombinase, mediating the rearrangement of immunoglobulin and T cell receptor genes [14]. Accordingly, information on DNA-PK activity in B cells would be of special interest in an autoantibody-producing disease like SS. Therefore, in the present work we set out to analyse if an altered stress reaction also appears in B lymphocytes from SS patients, by determining DNA-PK activity, p53 phosphorylation, cell cycle progression and apoptosis. Our results show that strong DNA-PK kinase activity in B-cell lines is part of the enhanced stress reaction in SS cells, probably contributing to our observations of heightened cell cycle arrest and apoptosis.

Materials and methods

Patients and cells

Peripheral venous blood was obtained from four SS patients and two randomly selected healthy blood donors at the Malmö University Hospital blood bank. The patients (three positive and one negative for SS-A/SS-B autoantibodies) fulfilled both the US–European and the Copenhagen classification criteria for SS [15, 16]. B-cell lines were established from each individual by Epstein–Barr virus (EBV) transformation as described [7]. We also used one EBV-transformed B line (RA 143) derived from a healthy individual, obtained from Dr A. Auerbach, Rockefeller University, New York, USA. HeLa cells (2-CCL) were from the American Type Culture Collection (Rockville, MD, USA).
All cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 25 mM HEPES and 12 μg/ml gentamicin at 37°C in a humidified 5% CO2 atmosphere.

The design of the work conformed to the standards currently applied in Sweden at the time of the study and each subject gave informed consent according to the Declaration of Helsinki before entering the study.

Ionizing irradiation

Gamma radiation was delivered by a neutron accelerator (Philips, Germany) at a dose rate of 0.70 Gy/min, at a distance of 50 cm from the cells, which were kept in their plastic culture flask in complete medium at room temperature. All cell cultures in the different assays were handled identically, the only difference being exposure to ionizing radiation. Following irradiation, cells were returned to the incubator and analysed later as indicated.

Extraction of nuclear proteins

Nuclear protein extracts were prepared by a modification of the procedure of Dignam et al. [17]. Cytoplasmic protein was removed by lysis of the cells in a low-salt buffer, and the sedimented cell nuclei were then lysed in a high-salt buffer, as described [10].

DNA-dependent protein kinase activity

The phosphorylating function of DNA-PK was measured using a synthetic p53 peptide (Promega, Madison, WI, USA) containing a DNA-PK specific phosphorylation site (serine 15) as substrate, as described previously [18]. Briefly, nuclear protein extract was mixed with [γ-32P]ATP and the synthetic p53 peptide, with or without sonicated DNA. The DNA-PK activity was calculated as counts per minute (c.p.m.) in the presence of DNA minus the c.p.m. in the absence of DNA. HeLa cells were included in each analysis and used as a calibrator in order to reduce the interassay variation. All nuclear extracts were analysed in triplicate, with an intra-assay coefficient of variation (CV) of 8.7%. The interassay CV, estimated using HeLa cells and determinations made on seven separate dates, was 19.4%.

Western blotting

A standard immunoblotting was performed as described [18]. Membranes were probed with the following antibodies: anti-Ku86 (p80, Ab-3) (clone S10B1) and anti-p53 (Ab-2) (clone PAb1801) from Oncogene (Cambridge, MA, USA); anti-DNA-PKcs (C-19) (SC-1552) and anti-p21(F-5) (SC-6246) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-ATM (AHP397) from Serotec (Serotec, UK). The peroxidase-conjugated secondary antibodies were from Dako (DAKO A/S, Denmark). The blots were developed using the enhanced chemiluminescence method (ECL; Amersham-Pharmacia, UK), together with X-ray film or a Bio-Rad Personal Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA).

Cell cycle analysis

For cell cycle analysis a Coulter Epics XL-MCL flow cytometer (Coulter, Miami, FL, USA) was used. According to a standard total DNA labelling protocol the cells were incubated on ice for 10–60 min in a hypotonic solution containing 0.01 M NaCl, 0.1% NP-40, 50 μg/ml propidium iodide and 10 μg/ml RNase A. To calculate the fractions of cells located in the different cell cycle phases MacCycle II software (Phoenix Flow, Phoenix, AZ, USA) was used and at least 8000 events were scored. For each individual three to six determinations were made at 24 h and one or two determinations 48 and 72 h after exposure to 15 Gy of irradiation.

Apoptosis

The same Coulter Epics XL-MCL flow cytometer was used, with filters detecting emission at 515–545 nm for fluorescein isothiocyanate (FITC) and 565–595 nm for propidium iodide. Background autofluorescence was monitored for each sample by analysing unlabelled cells. Apoptosis was measured as described [19], and at least 3000 events were scored. For each individual, two or three determinations were performed in unirradiated and 24 h post-irradiation cells. FITC-labelled Annexin V was from Alexis (Switzerland) and PharMingen.

Statistical analysis

The Mann–Whitney U-test was used for statistical analysis of the DNA-PK activity data, which included results from three controls and four SS patients. The lack of overlap between these two groups yielded a P value of less than 0.05 (0.028), considered statistically significant.

Results

High level of DNA-dependent protein kinase activity in SS

EBV-transformed B-cell lines were exposed to gamma radiation, post-incubated for 24 h under optimal growth conditions, and then subjected to nuclear protein extraction. DNA-PK kinase activity was determined by the incorporation of radiolabelled ATP into a synthetic p53 peptide containing a DNA-PK phosphorylation site. DNA-PK activity was calculated by subtracting the amount of radioactivity incorporated in the absence of sheared DNA from that incorporated in the presence of DNA. The assay takes advantage of the observation that DNA-PKcs appears to be the major DNA-activated kinase in mammalian cells [20]. The DNA-PK activity was higher in all four SS patients than in all three controls; i.e. there was no overlap between the groups (P = 0.028) (Fig. 1). The increased activity in SS was significant at all radiation doses, including unirradiated cells. There was no sign of induction of DNA-PK activity due to radiation exposure (except for the SS3 patient at 2 Gy); instead, a dose-dependent reduction was seen.

Normal Ku86 and DNA-PKcs protein content in SS

The nuclear protein extracts were also used for Western blotting semiquantitation of Ku86 and DNA-PKcs protein content. SS and control samples, at each radiation dose, were electrophoresed on separate immunoblotting gels. However, it has been shown by representative experiments in Fig. 2. These data do not permit evaluation of the influence of radiation on the protein level, since the extracts representing different radiation doses were analysed on separate immunoblotting gels. However, it has been reported that DNA-PK shows no inducibility in response to DNA damage [21].

Enhanced cell cycle arrest due to gamma radiation in SS

At 24, 48 and 72 h after 15 Gy of irradiation, samples were drawn from the B-cell cultures and the DNA content was analysed by flow cytometry in order to determine cell cycle progression.
Multiple facets of the stress response are enhanced in SS

An overview of the results of the present study is attempted in Table 1, by assigning a ranking number to each normal and SS individual, number 1 denoting the strongest defence reaction. The SS patients hold all the top positions in the pre-irradiation defence functions (DNA-PK kinase activity and apoptosis) as well as in the radiation-induced cell cycle arrest response (Table 1).

Discussion

Human cells express a number of complex and interacting mechanisms that constitute the stress response to DNA damage, including cell cycle arrest, allowing time for enzymatic DNA repair, and apoptosis of severely damaged cells. Some molecular components of these defence functions are expressed constitutively, whereas others become induced upon DNA damage formation. Double-stranded DNA breaks are thought to arise continuously in all cells, due to, for example, environmental radiation and endogenous radical oxygen species. Even a low frequency of this potent damage type, perhaps a single lesion per cell, is capable of inhibiting DNA replication and may threaten cellular homeostasis unless a stress response is evoked [5]. For the present work on the stress response in SS, we decided to focus on B cells and on gamma radiation-induced damage. Double-stranded DNA breaks occur physiologically in B cells during the rearrangement of immunoglobulin genes, and the DNA-PK complex participates in this recombination, not only in the bone marrow but also in mature B cells responding to antigen

Enhanced apoptosis in SS B cell lines

Early apoptosis was analysed by flow cytometry of the same radiation-exposed B-cell cultures as in the cell cycle experiments, and defined by the binding of annexin V to cells impermeable to propidium iodide. Results from representative experiments are visualized by the annexin V-positive but propidium iodide-negative events of the dot plot in the lower right quadrant (Fig. 6). The controls display a result similar to that reported in the literature for normal individuals, with a pre-irradiation value of approximately 10% apoptotic cells and becoming significantly increased by gamma radiation [23]. Two of the three SS lines differed markedly from this normal pattern even before irradiation, by having high median values for apoptotic cells (24 and 36% respectively) (Table 1). At 24 h after irradiation, the higher apoptosis frequencies were found in the SS cases (medians of 34–71%) than in the controls (33–35%). There was no apparent difference between SS and healthy controls in radiation-induced apoptosis; however, such a comparison is complicated by the very high apoptosis frequency (up to 71%) in irradiated SS cells (Table 1).

Normal p53, p21 and ATM protein levels in SS

p53 is known to activate transcription of p21, leading to inhibition of cell cycle progression from the G1 phase. Western blotting of nuclear protein extracts revealed induction of p53 and p21 upon gamma-irradiation in all cells examined; this is in accordance with the observation of G1 cell cycle arrest. ATM expression was unaffected by radiation, in agreement with the reported lack of inducibility of ATM [21]. There was no apparent difference between SS and healthy controls in the protein levels, as shown by a representative experiment in Fig. 5. However, it should be emphasized that this result does not exclude a difference in biological activity of these DNA damage response modifiers; compare our DNA-PK results in Figs 1 and 2 showing no effect on protein level but higher kinase activity in SS cells.

Representative DNA histograms generated by the MacCycle software are shown in Fig. 3. The gamma radiation-induced response by the healthy controls is in agreement with that reported in the literature for EBV-transformed B-cell lines obtained from normal individuals [22, 23]. The reduction of cells located in G1 phase should be interpreted to result from two opposing influences: a G1 arrest function (under the influence of p53), together with a relatively strong loss of in-flow into G1 [24] resulting from DNA damage inhibiting DNA replication. This interpretation is supported by the findings of an intensified loss of G1 cells from ataxia telangiectasia (AT) patients known to be defective in ATM protein and cell cycle arrest function. Interestingly, cells from all the SS patients show a marked difference from the normal cells, contrasting with the abnormality reported in the AT cells, with a retention of G1 cells in response to gamma radiation (Fig. 4). This was observed at 24, 48 and 72 h after exposure to radiation. As expected, radiation can be seen to increase the fraction of sub-G1 events; i.e. cells or cell debris containing less than one DNA copy number. Some of these events can be assumed to represent cell death; see below for the apoptosis assay.
in lymph nodes [25]. Moreover, DNA-PK is essential for the repair of double-stranded DNA breaks by the mechanism of non-homologous end-joining, occurring mainly in G1, and also for the activation of the G1 arrest function mediated by p53 accumulation and p21 gene expression [5].

Accordingly, if a disease (such as SS) is found to have abnormalities in both immune regulation and stress response, we reasoned that the molecular basis may be shared between these two processes, DNA-PK being a likely candidate. EBV-transformed, continuously growing B-cell lines were used, since sufficient peripheral blood B cells could not be obtained from patients. In a previous study, Zeher et al. [26] found that the increased susceptibility to apoptosis of peripheral blood CD4+ T cells from SS patients correlates with lymphocyte activation. We find it unlikely that our EBV lines, cultivated for several weeks and for more than ten generations in vitro at the time of these experiments, should retain an imprint of in vivo activation status. Therefore, we considered it wise to avoid primary B cells, acknowledging that SS-related in vivo activation of B cells might influence any result in a stress response study.

To what extent is the cellular stress response affected by the status of EBV transformation and its accompanying growth alterations? Infection of human B lymphocytes with EBV in vitro induces a G0 to G1 transition followed by DNA synthesis and cell division. This cell cycle activation closely mimics the antigen-dependent normal B-cell activation pathway and is mediated by the virus-encoded latent membrane protein 1 (LMP1), which is a functional homologue of CD40, and LMP2, which mimics the

FIG. 3. Cell cycle progression in gamma-irradiated B cells. DNA content was analysed by flow cytometry of propidium-labelled permeabilized cells. Representative experiments using cells incubated for 24 h after exposure to radiation are shown, with DNA histograms generated by the MacCycle software, referring the cells to cell cycle phases. The resulting value for the percentage of cells located in G1 is inserted into each histogram.

FIG. 4. Increased G1 cell cycle arrest in SS patients. The fraction of cells located in the G1 phase, at the indicated times after exposure to radiation, was determined by flow cytometry, as shown in Fig. 3. Each symbol represents one individual, and shows the median value from three to six determinations at 24 h and one or two determinations at 48 and 72 h. Open circles, unirradiated controls; filled circles, unirradiated patients; open triangles, irradiated controls; filled triangles, irradiated patients.

FIG. 5. Western blot determination of p53, p21 and ATM protein levels in gamma-irradiated and 24 h post-incubated B cells. Nuclear protein extracts were separated by SDS-PAGE and immunoblotted. Results from representative experiments are shown. Samples from all three radiation doses for each individual were electrophoresed on the same gel, facilitating evaluation of the influence exerted by radiation.
**TABLE 1. Enhanced stress response functions in SS, illustrated by ranking**

<table>
<thead>
<tr>
<th>Individual</th>
<th>DNA-PK activity</th>
<th>G1 arrest</th>
<th>Apoptosis</th>
<th>DNA-PK activity</th>
<th>G1 arrest</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>5 (57)</td>
<td>n.a.*</td>
<td>4 (5)</td>
<td>n.a.*</td>
<td>4 (24)</td>
<td>3 (31)</td>
</tr>
<tr>
<td>C2</td>
<td>6 (33)</td>
<td>n.a.*</td>
<td>4 (5)</td>
<td>n.a.*</td>
<td>5 (32)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>C3</td>
<td>7 (28)</td>
<td></td>
<td></td>
<td>n.a.*</td>
<td>1 (2)</td>
<td>5 (26)</td>
</tr>
<tr>
<td>SS1</td>
<td>2 (66)</td>
<td>n.a.*</td>
<td>3 (7)</td>
<td>n.a.*</td>
<td>3 (15)</td>
<td>1 (42)</td>
</tr>
<tr>
<td>SS2</td>
<td>3 (65)</td>
<td>n.a.*</td>
<td>2 (24)</td>
<td>n.a.*</td>
<td>2 (12)</td>
<td>2 (35)</td>
</tr>
<tr>
<td>SS3</td>
<td>4 (58)</td>
<td></td>
<td></td>
<td>n.a.*</td>
<td>1 (36)</td>
<td></td>
</tr>
<tr>
<td>SS4</td>
<td>1 (88)</td>
<td>n.a.*</td>
<td>1 (36)</td>
<td>n.a.*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ranking of all SS patients and control subjects according to the results for stress response functions. The rank number is given in bold type. Figures in parentheses are median values: for DNA-PK activity, the c.p.m. × 1000 for p53 phosphorylation; for cell cycle arrest, the reduction of % cells in G1 phase caused by radiation at 48 h post-incubation; for apoptosis, the percentage of annexin V-positive, propidium iodide-negative cells 24 h after irradiation.

*n.a., not analysed. Cell cycle arrest, by definition, cannot be determined in unexposed cells.

**No induction of DNA-PK activity due to gamma radiation was seen.**
immunoglobulin rearrangement, our findings raise the question of whether the DNA-PK alteration influences B-cell maturation and, thus, the development of autoimmune features. B-cell activating factor, secreted by macrophages and dendritic cells, has recently become strongly linked to the development of SS in both man and mice [39]. It is therefore intriguing to consider whether an elevated stress response is a primary aetiological factor in SS [11], leading to hyper-alert innate immunity, and also to derangement of adaptive immunity, as predicted by the Danger model for lymphocyte activation [40].

The authors have declared no conflicts of interest.

References