Abstract

The two L5178Y (LY) sublines bear a heterozygous Tp53 mutation that affects its transactivation function. LY-S (radiation-sensitive) cells are deficient in double strand break (DSB) repair by non-homologous end-joining (NHEJ) and do not express p21WAF1 (Cdkn1a) either constitutively or after x-irradiation, in contrast to their radiation-resistant counterpart LY-R cells, which express p21WAF1 constitutively. Radiation-induced G2 arrest in LY-S cells is very long (11 h/Gy) but 2 mM caffeine treatment shortens it, decreases the fraction of G2 cells and increases the fraction of apoptotic cells. The treatment also increases the DNA damage that is estimated with the comet assay 18 h after irradiation with 5 Gy (ca. 23% of the initial value for x-rays and ca. 47% for x-rays plus caffeine). This indicates that either the repair has not been completed or the apoptotic DNA fragmentation has been initiated (or both). The same treatment applied to x-irradiated (5 Gy) LY-R cells (G2 arrest, 4 h/Gy) has no radiosensitising effect, induces no apoptosis and does not alter the amount of DNA damage left unrepaired (ca. 28%). The results are compatible with the assumption that inhibition of the Atm-dependent homologous recombination repair by caffeine, brings differential effects in LY sublines because of the defect of the alternative DNA repair system (NHEJ) in LY-S cells.

Introduction

Prolonged G2 arrest is a defence mechanism that protects cells with unrepaired DNA from entry into mitosis. A pair of human cancer cell lines (HeLa, cervical carcinoma and MeWo, melanoma) [1] and the H-reg and v-myc transfected rat embryo fibroblasts (REF) [2], are good examples of that protective effect. Radiation resistance corresponds with longer G2 arrest, and the prolonged (as compared to untransfected REF) G2 arrest also protects from apoptosis [2]. Hence, a treatment that shortens the G2 arrest can be expected to radiosensitise cells; one effect is an increase in the fraction of apoptotic cells, which are then eliminated from the irradiated population. Such an effect is usually seen after caffeine treatment of x-irradiated cells [3].

It has recently been found that caffeine inhibits the kinase activity of ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia related) proteins [4, 5]. This explains its action on the G2 arrest [6], since the phosphorylation pathway from ATM and ATR leads through Chk1, 2 and Cdc25C phosphatase; the latter, when phosphorylated, does not activate the cyclin B1/Cdc2 complex thus causing G2 arrest. This mechanism is “switched on” as long as the unrepaired DNA damage is signalled by ATM and ATR. Caffeine gives a false signal of completed repair by inhibiting ATM and ATR kinase activity, the cyclin B1/Cdc2 complex becomes activated and the cell is prepared to enter mitosis.

There is a redundancy in G2 arrest control in irradiated mammalian cells [7]. Cdc25C can be sequestered in the cytoplasm by the 14–3-3-σ protein, whose expression is under the transcriptional control of Tp53 [8]. Transcription of cyclin B is under control of Tp53 [9], and moreover, the kinase inhibitor, p21WAF1 (now also termed Cdkn1a), induced by Tp53 in response to DNA damage directly inhibits the cyclin B/Cdc2 complex [10]. These G2 arrest control mechanisms are diagrammatically outlined in Fig. 1.

The duration of the radiation-induced G2 arrest in mammalian cells may be expected to be directly related...
to the proportion of DNA damage left unrepaired [11], although no general relationship of these two parameters to radiation sensitivity could be found (e.g. [12], also reviewed in [13]). Nevertheless, the response to x-rays of L5178Y (LY) sublines which differ in radiation sensitivity and double strand break repair competence [14, 15, 16, 17, 18] meets this expectation very well. The radiation-sensitive variant, LY-S, has an exceptionally long mitotic delay (11 h/Gy), whereas the parental, more radiation-resistant LY-R line, requires about 4 h/Gy [15]. The mean lethal doses of x-rays are about 1 Gy and 0.5 Gy for LY-R and LY-S cells, respectively [15].

Both L5178Y sublines are heterozygous for a Tp53 mutation (TGC → CGC; codon 170) [19]. Mutation at this site impairs the function of the Tp53 DNA binding domain and thus, indirectly, its transactivating capacity (reviewed by Levine [20]). The transactivation-deficient Tp53 mutation is usually associated with a weaker apoptotic response, nevertheless, a high level of a transcriptionally incompetent Tp53 can induce apoptosis [21, 22]. Although the two LY sublines examined here are heterozygous for the Tp53 mutation, there is a difference between them in the extent of the delayed apoptosis triggered by x-irradiation. Post-irradiation apoptosis is accompanied by a weak and delayed accumulation of Tp53 protein and lack of changes in Bax, BclxL or Bcl2 expression in the radiation-sensitive LY-S subline, as compared to the radiation-resistant one (LY-R) [23]. The main reason for the high radiation sensitivity of LY-S cells is a deficiency in DNA double strand break (DSB) repair [16, 17], apparently caused by impaired non-homologous end-joining (NHEJ) [18], whereas homologous recombination repair is functional [24].

The effect of radiation plus caffeine treatment in LY sublines was first examined in 1984 [25]. Here, we re-examine it to check how impaired NHEJ and mutated p53 affect the control of radiation-induced G2 arrest.

**Materials and methods**

**Cell cultures**

The murine leukaemic lymphoblasts LY-R and LY-S were maintained in suspension cultures in Fischer’s medium supplemented with 8% heat-inactivated bovine serum [26]. Asynchronous populations in an exponential phase of growth were used in all experiments.

**Caffeine treatment**

Cell cultures were diluted 1:1 with sterile 4 mM caffeine solution in Fischer’s medium just before irradiation or sham-irradiation. Caffeine was present in the medium until the end of the incubation.

**X- and γ-irradiation**

X-irradiation was performed with the use of a Stabilipan-250 instrument (Siemens, Erlangen, Germany). The conditions of irradiation were 180 kV, 18 mA, 1 mm copper filter and a dose rate of 1.28 Gy/min. Cell cultures (10 ml aliquots, 8 × 10^6 cells/ml) were irradiated at 0°C and kept in an ice-water bath until the last portion was irradiated. They were diluted twice with Fischer’s medium (20°C) and placed in a CO2 incubator at 37°C. Zero time samples were processed immediately without dilution, control samples were treated identically, only omitting the irradiation step.

For determination of p21, gadd45, Jnk and Erk, 200 ml aliquots of cell cultures (4 × 10^6 cells/ml) were gamma-irradiated at 0°C with the use of a 60Co gamma source (MINEOLA) at a dose rate of 60.5 Gy/h.

**Polymerase chain reaction (RT-PCR)**

Total RNA was isolated by the method of Chomczynski and Sacchi [27] using Trizol reagent (GIBCO BRL). First strand cDNA synthesis was primed using the Superscript premplification system (GIBCO BRL) with oligo(dT). The cDNA was amplified using specific primers. The primer sequences, annealing temperatures and product size were:

- **p21/WAF1**: 5' - GGCGGCGAGACACGATGAGTT-3'
- **Gadd45**: 5' - GAGAGGGGGCCCTGTGAGTT-3'
- **p21, gadd45**: 5'-GGAGGGCGGGCCCTGTGAGTTGAGTGCC-3'
- **p21**: 5'-GGCGGCCGCGTTGAGTT-3' (63°C, 396 bp)
- **Gadd45**: 5'-GGAGGGCGGGCCCTGTGAGTTGAGTGCC-3' (60°C, 385 bp)
- **p21, gadd45**: 5'-GGCGGCCGCGTTGAGTT-3' (63°C, 396 bp)

We used the following PCR conditions: denaturation at 95°C for 5 min, 31 cycles at 95°C for 1 min, at the appropriate annealing temperature for 1 min, 72°C for 1 min and an elongation step at 72°C for 10 min. Probes were separated by electrophoresis in 2% agarose and visualised in UV-C after staining with ethidium bromide.

**Western blotting**

For cyclin B1, c-Abl, c-Myc and c-Fos detection, control and irradiated cell cultures (3 × 10^6 cells) were incubated for 2 or 24 h and harvested for extract preparation according to the research application manual of Santa Cruz Biotechnology, with the use of protease inhibitor cocktail tablets (Boehringer Mannheim). For Erk and Jnk detection the incubation times were 2 h and 4 h. Cell extract proteins were separated on 10% SDS-PAGE gels and 60 µg aliquots of protein were loaded. The gels were run at 200 V for 30–35 min, using the Bio-Rad Apparatus (Mini) and electrotransferred from the gel to nitrocellulose membranes (Amersham International). Blots on nitrocellulose membranes were analysed with monoclonal or polyclonal antibodies from Santa Cruz Biotechnol-