The dynamic nature of DNA-strand breaks present in differentiating muscle cells and quiescent lymphocytes

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Cellular differentiation in a number of eukaryotic systems is associated with changes in the number of DNA-strand breaks and involves the activity of adenosine diphosphoribosyl transferase (ADPRT). DNA-strand breaks are essential for activation of nuclear ADPRT, the activity of which is required for efficient religation of DNA-strand breaks. In this study we demonstrate the dynamic nature of DNA-strand breaks formed in the genome of differentiating avian skeletal muscle cells and quiescent human lymphocytes. Inhibition of ADPRT activity blocks DNA-strand ligation in both cell types and leads to the accumulation of a higher number of strand breaks.

DNA-strand break *ADP-ribosylation* *Differentiation* *Mitogen activation*

1. INTRODUCTION

Single-strand DNA breaks are formed at an early stage during the spontaneous differentiation of primary avian skeletal myoblasts in culture [1,2]. Induction of differentiation in murine erythroleukaemic cells [3,4], human promyelocytic leukaemia cells [5] and normal human granulocyte-macrophage progenitor cells [6,7] also results in the early formation of DNA-strand breaks. Quiescent human and murine lymphocytes contain DNA-strand breaks which are ligated soon after mitogen stimulation (2–20 h), before most indications of activation are manifest [8–10]. In all of these examples alteration in the number of DNA-strand breaks is correlated with the requirement for adenosine diphosphoribosyl transferase (ADPRT) activity (review [11]). The activity of the nuclear ADPRT (recent reviews [12,13]), which is dependent on the presence of DNA-strand breaks [14], is required for efficient DNA excision repair because it regulates the ligation of DNA-strand breaks [15], probably through the modulation of DNA Ligase [16,17]. A requirement for ADPRT activity has also been shown in the differentiation of such diverse eukaryotic systems as the protozoan parasite *Trypanosoma cruzi* [18,19] and for the expression of foetal enzymes by cultured adult rat hepatocytes [20].

To further characterise the DNA-strand breaks which are formed in the course of differentiation of avian skeletal muscle cells and human lymphocytes we have investigated the effect of ADPRT inhibitors on these breaks. Here we describe the effect of a competitive inhibitor of ADPRT activity, 3-aminobenzamide [21], on the steady-state number of DNA-strand breaks present in the lymphoid and muscle cells at various stages of their differentiation. The results show that the

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Abbreviations: ADPRT, adenosine diphosphoribosyl transferase (EC 2.4.2.30); PHA, phytohaemagglutinin

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differentiation-related breaks detected in earlier studies [2,5,8–10] are not static but rather are the net steady-state result of a dynamic equilibrium of formation and ligation of DNA-strand breaks.

2. MATERIALS AND METHODS

2.1. *Muscle cell cultures*

Primary chick myoblasts were isolated from the thigh muscle of 12-day-old chick embryos and cultured as previously described [2]. To study the effect of ADPRT inhibition on the formation and ligation of DNA-strand breaks, which appear during myoblast differentiation [2], cultures were maintained in either the continuous presence of 8 mM 3-aminobenzamide, a competitive inhibitor of ADPRT activity [21], or 8 mM 3-aminobenzamide was added 2.5 h prior to the harvesting of the cells, using a rubber policeman, in phosphate-buffered saline.

2.2. *Lymphocyte cultures*

Peripheral blood lymphocytes were isolated [10] from healthy human donors using dextran depletion of erythrocytes followed by density-gradient centrifugation on Ficoll-Plaque (Pharmacia). The cells (10^6/ml) were stimulated with 2.5 μg/ml of phytohaemagglutinin (PHA; leucoagglutinin, Pharmacia) in RPMI 1640 medium containing 5% foetal calf serum in conical-bottomed 16 × 110 mm tubes (800 μl/tube) at 37°C in an atmosphere of 5% CO_2. Parallel cultures were also set up of T-lymphoblastoid cell lines, MOLT 4 and CEM, and of unstimulated peripheral lymphocytes, without mitogen. After 16–20 h, either 5 mM or 8 mM 3-aminobenzamide (see table 1) was added to half of the tubes in each batch and the incubation was continued for a further 4 h. 10 ml phosphate-buffered saline was then added to each tube and the cells were pelleted (200 × g, 5 min). The nucleoid sedimentation rate was then estimated as described below.

2.3. *Measurement of DNA-strand break formation and ligation*

DNA-strand breaks were measured by estimating the nucleoid sedimentation rates [22,23] in neutral sucrose gradients containing high salt and a non-ionic detergent as described [2,8]. Briefly, cells were lysed directly on the gradients in a solution containing 2 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 8.0) and 0.5% (v/v) Triton X-100. The linear sucrose gradients (15–30% for myoblasts and 5–20% for lymphocytes) also contained 2 M NaCl, 10 mM EDTA and 10 mM Tris-HCl (pH 8.0), but no detergent. Where indicated 30 μg/ml ethidium bromide was included in both the lysis solution and the sucrose gradient to avoid possible effects on the nucleoid sedimentation rates of changes in chromatin composition [24,25]. The gradients were kept in the dark for 15 min at 20°C and then centrifuged at either 10000 rpm in a Beckman SW50.1 rotor for 45 min (muscle cells) or 25000 rpm in a Beckman SW41 rotor for 30 min (lymphocytes). The position of the nucleoids was then identified by collecting the gradients from the bottom through the flow cell of a recording spectrophotometer set at 260 nm or by direct identification of the nucleoid band against a UV light source in those instances in which the gradient contained ethidium bromide [25].

3. RESULTS AND DISCUSSION

Myoblasts continued to proliferate during the first 24–36 h of culture under the conditions employed. At about 40 h muscle cell fusion began and by 72 h approx. 70 ± 8% of the cells had fused to form the multinucleated syncytia of muscle fibres. As previously demonstrated DNA-strand breaks are formed during the spontaneous differentiation of primary chick myoblasts in culture [2]. This is reflected in the higher sedimentation rate of nucleoids from proliferating, undifferentiated, myoblasts at 18 and 26 h of culture compared to the slower sedimentation rate of nucleoids from differentiating muscle cells at 40, 64 and 92 h of culture (fig.1, ●). We have demonstrated that the formation of these breaks is not due to a general deficiency in the DNA repair mechanisms of the terminally differentiating muscle cells, as judged by comparison of the kinetics of strand ligation after irradiation of cells at various stages of differentiation [2]. The inhibition of ADPRT activity by the presence of 3-aminobenzamide either continuously (fig.1, ▲) or for 2.5 h prior to estimation of the nucleoid sedimentation rate (fig.1, ■) has little effect on the nucleoid sedimentation rate before the onset of terminal differentiation at about 40 h. However, ADPRT inhibition

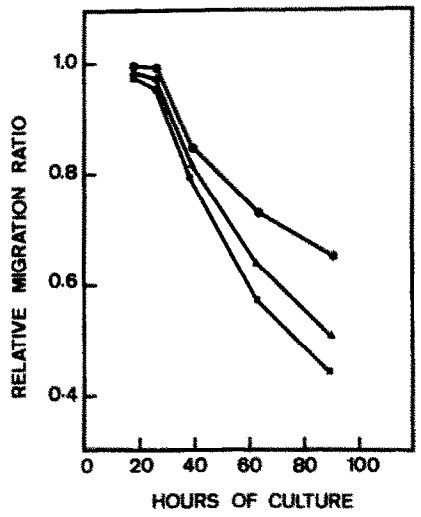

Fig.1. Effect of inhibition of ADPRT activity on the nucleoid sedimentation rate of differentiating muscle cells. Muscle cells were cultured in the absence of an ADPRT inhibitor (●), or in the presence of 8 mM 3-aminobenzamide either continuously (▲) or for 2.5 h prior to nucleoid sedimentation rate analysis (■). The rate is expressed as the ratio of migration of nucleoids from cultures at the indicated times to nucleoids from 18 h proliferating cultures. These gradients contained 30 μg/ml ethidium bromide (see section 2).

reduces the sedimentation rate of nucleoids from differentiating cultures (fig.1). Consistent with previous observations [13], these results suggest that inhibition of ADPRT activity blocks not the formation but the religation of DNA-strand breaks which appear during myoblast differentiation.

The effect of the inhibition of ADPRT activity on the number of DNA-strand breaks was also investigated in lymphocytes at various stages of activation. Table 1 shows the results obtained from 5 separate experiments of a 4 h incubation in 3-aminobenzamide on the migration of nucleoids from unstimulated lymphocytes, mitogen-activated lymphocytes and lymphoblastoid cell lines. As reported [10] quiescent lymphocytes appear to have more DNA breaks than the PHA-activated cells or the lymphoblastoid cell lines, indicated by the slower sedimentation of their nucleoids. Like the differentiating muscle cells (see above), these breaks are not symptomatic of a deficiency in DNA repair mechanisms since quiescent lymphocytes are well known for their proficient repair capabilities and we have confirmed this in our own studies (unpublished). The nucleoid sedimentation rate of only the unstimulated cells was slowed significantly by treatment with 3-aminobenzamide.

Table 1

Effect of 3-aminobenzamide on the migration of nucleoids from lymphoid cells

Experiment number	Quiescent lymphocytes with 3-aminobenzamide	Stimulated lymphocytes	Stimulated lymphocytes with 3-aminobenzamide	Lymphoblastoid lines	Lymphoblastoid lines with 3-aminobenzamide
Without ethidium bromide:					
1	0.79 (0.79)	1.15	1.06 (0.92)	1.34	1.34 (1.00)
2	0.81 (0.81)	1.18	1.14 (0.97)	1.27	1.26 (0.99)
3	0.87 (0.87)	1.25	1.25 (1.00)	1.30	1.19 (0.92)
Mean ± SE of experiments 1–3	(0.83 ± 0.02)		(0.94 ± 0.03)		(0.97 ± 0.02)
With ethidium bromide:					
4	0.77 (0.77)	1.25	1.16 (0.94)	n.d.	n.d.
5	0.80 (0.80)	1.12	1.12 (1.00)	n.d.	n.d.

Quiescent lymphocytes, lymphocytes after 16–20 h stimulation with PHA, or lymphoblastoid cell lines CEM and MOLT4 were incubated in the presence or absence of 3-aminobenzamide (8 mM for experiment 1, 5 mM for remainder) for 4 h at 37°C and the migration of their nucleoids determined in the presence or absence of ethidium bromide (see section 2). Results are expressed as migration relative to quiescent lymphocytes (1.00) and, in parentheses, as migration with inhibitor/migration without inhibitor for each cell preparation. The figures for experiments 1–3 are the mean of duplicate assays and the figures for experiments 4 and 5 are the mean ± SE; n.d., not determined

The inhibitor affected mitogen-activated lymphocytes much less and the lymphoblastoid cell lines incubated with 3-aminobenzamide had a sedimentation rate the same as nucleoids from non-inhibited cells. Similar results were also obtained in 2 experiments when the nucleoid analyses were carried out in the presence of ethidium bromide. Greer and Kaplan [26] have reported that inhibition of ADPRT activity by 5 mM 3-methoxybenzamide causes an increase in the number of DNA-strand breaks in both resting and Con A activated murine lymphocytes. However, this was only evident after 11 h incubation under conditions resulting in 4–10% cell death (the methoxy derivative of benzamide is more toxic than the amino) and the breaks continued to increase for the length of the experiment (23 h). In our lymphocyte system, culturing quiescent cells in the presence of the inhibitor for various times showed that the effect on nucleoid sedimentation rate was constant (0.81 ± 0.04) between 2 and 20 h (the shortest and longest times investigated). We interpret this data as indicating the perturbation of the equilibrium position of a dynamic system.

Our data from these 2 diverse systems demonstrate that inhibition of DNA-strand break ligation (by inhibiting ADPRT activity) increases the steady-state number of DNA-strand breaks which are formed both during myoblast differentiation and in quiescent lymphocytes. However, inhibition of ADPRT activity does not increase the steady-state number of DNA-strand breaks in undifferentiated myoblasts, mitogen-stimulated lymphocytes or in lymphoblastoid cell lines. Thus the increase in DNA-strand breaks occurs only when physiologically formed breaks are present. Inhibition of ADPRT activity by 3-aminobenzamide, at the concentrations used here, affects neither cell viability nor growth of myoblasts [2] or activated lymphocytes [10]. It has already been demonstrated that inhibition of ADPRT activity does not directly cause or affect the formation of DNA-strand breaks but does block the ligation of breaks formed during DNA excision repair [13]. Therefore, the DNA-strand breaks present in differentiating muscle cells and in quiescent lymphocytes are the net product of a dynamic state of DNA-strand break formation and ligation. Hence the inhibition of both myoblast differentiation and of the activation of mitogen-stimulated lymphocytes by inhibitors of ADPRT activity may be caused by the inhibition of DNA-strand break ligation which normally occurs during these processes.

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