Mechanisms of Glucocorticoid-Induced Growth Inhibition and Cell Lysis in Mouse Lymphoma Cells

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A. Introduction

Glucocorticoid hormones are known to have a wide variety of molecular effects by induction or repression of proteins at the transcriptional level via a receptor system. Glucocorticoids, depending on the nature of the target tissue, can not only regulate carbonhydrate, protein and nucleic acid metabolism but can also accelerate or inhibit cellular growth or differentiation. Among the cell types that involute during prolonged exposure to steroids are certain lymphocytes and lymphoma cells. This effect is the basis of steroid therapy for malignant lymphomas. The steroid-induced death of lymphoma cells has morphological characteristics clearly distinct from necrosis, i.e., early alterations of the nuclear structure together with progressive reduction of the cellular volume but preservation of the integrity of cytosolic organelles [1] which are altered early during necrosis. This second type of cell death, called apoptosis, represents an active process involving an alteration of the pattern of active genes [2] in contrast to necrosis which is the consequence of environmental perturbations.

While the therapeutic potential of corticosteroids has been known for many years, the molecular basis of the steroid-triggered cytolysis of lymphoma cells has not been elucidated. In this contribution, an overview of the possible molecular events leading to the lysis of S49.1 murine lymphoma cells as well as the implications for the therapy of malignant lymphomas are presented.

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B. Two Phases of Glucocorticoid Action on Lymphoma Cells

S49.1 lymphoma cells exposed to the synthetic glucocorticoid dexamethasone respond to the steroid challenge by various metabolic alterations. Among the earliest effects is a reduction in the rate of glucose and amino acid transport [3], followed by a decrease in the rate of DNA, RNA and protein synthesis [4, 5]. These findings suggest that the first action of glucocorticoids is to switch cells from the proliferating to the nonproliferating state. This initial steroid effect is reflected by an accumulation in G_1 of the cell cycle [6]. This was confirmed by the experiment shown in Fig. 1. Glucocorticoid-induced cell death is initiated after a lag phase of about 24 h (Fig. 1 A). During this time the steroid effect is fully reversible, i.e., when the glucocorticoid was removed and the cells cloned in soft agar no reduction in the colony forming ability was detectable before 24 h (Fig. 1 B). In contrast, when lymphoma cells were treated with glucocorticoids, washed at various times to remove the steroid, and incubated further until day 3 after hormone addition, a pronounced decrease of the growth rate was detectable even when the cells were exposed to the steroid for only 6 h (Fig. 1 C). Consequently, two phases of glucocorticoid action on lymphoma cells can be discriminated, a reversible cytostatic phase followed by an irreversible cytolytic phase. This finding sug-

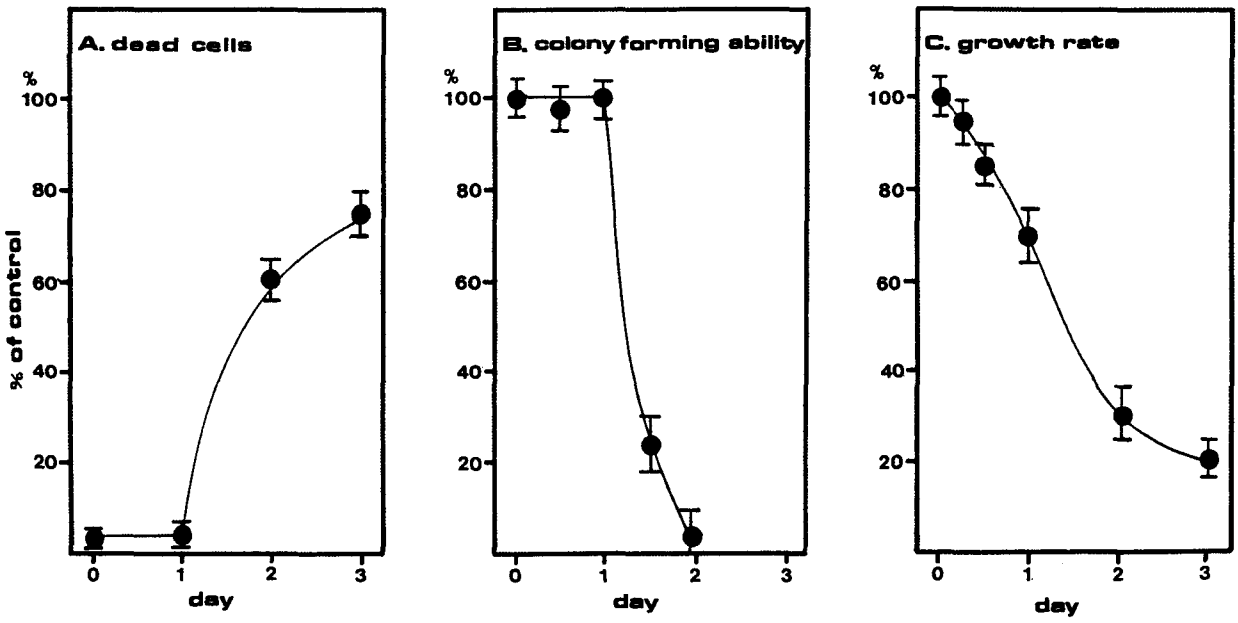

Fig. 1A–C. Cell death (**A**), colony forming ability (**b**) and growth rate of dexamethasone-treated lymphoma cell cultures (**C**). S49.1 lymphoma cells were seeded and treated with 10^{-7} *M* dexamethasone. At the times indicated the cell viability (**A**) of dexamethasone-treated or control cultures was determined, or the cells were washed and reseeded at the same densities and the colony forming ability (**B**) was measured, or the number of living cells was monitored on the third day after steroid addition (**C**) as described in [7]

gests that the two processes are the result of distinct molecular mechanisms.

Since steroid "toxicity" depends on the growth rate, with a high number of lysed cells appearing under suboptimal growth conditions versus a lower number under optimal conditions [7], one can speculate that steroid-mediated growth inhibition may be brought about by interference with the growth-regulating system, either directly or indirectly by depression of the synthesis of autocrine growth factors.

C. Autocrine Growth factors of S49.1 Lymphoma Cells

Dilution of S49.1 cell cultures drastically reduces the growth rate, an effect which can be reversed by addition of conditioned medium and which points to the dependence of lymphoma cell proliferation on autocrine growth factors [7]. The nature of the factor(s), however, remains as yet unknown. Neither interleukin 2 nor other interleukins tested, including interleukins 1, 3 and 4, showed growth stimulating potential (Wielckens, unpublished observations).

Charcoal treatment of conditioned medium, however, revealed that one of the growth promoting factors is a small molecule with high affinity to charcoal. Since metabolites of arachidonic acid which bind strongly to activated charcoal have been shown to promote growth of cultured cells [8, 9] we first tested whether at least one of the autocrine growth factors is derived from arachidonic acid. Since the nonspecific lipoxygenase inhibitor nordihydroguaiaretic acid completely blocked S49.1 cell proliferation, in contrast to the cycloxygenase inhibitor indomethacin, the growth factor appeared to be a product of the lipoxygenase pathway. Of the metabolites tested (5-hydroxyeicosatetraenoic acid = 5-HETE, 8-HETE, 9-HETE, 12-HETE, 15-HETE, lipoxins A and B, leukotrienes B_4, C_4, D_4 and E_4), only leukotriene B_4 could restore the reduced growth rate in lymphoma cell cultures with low cell numbers, suggesting a critical role of this factor under the test conditions [7]. Even

the precursor arachidonic acid did not significantly affect lymphoma cell proliferation. Furthermore, it was shown that S49.1 cells synthesize nanogram amounts of leukotriene B_4, arguing for it having a role as an autocrine growth factor [7].

As suggested by the lack of a pronounced effect of leukotriene B_4 on colony-forming ability in soft agar, however, S49.1 lymphoma cell growth appears not to depend only on this arachidonic acid metabolite. When cells are seeded at a still higher dilution than in the low density cell multiplication assays, the concentration of other essential autocrine growth factors may fall under a critical level. Therefore, it can be concluded that the diluted cell cultures still contain enough of other growth factors necessary to sustain lymphoma cell proliferation. Preliminary experiments suggest that at least two additional growth promoting factors are present in conditioned medium, one probably a protein, the other possibly a small molecule derived from arachidonic acid.

The antiinflammatory effect of corticosteroids has been attributed to inhibition of arachidonic acid release by induction of a phospholipase A_2 inhibitor, thereby blocking the formation of prostaglandins, leukotrienes and other hydroxyeicosatetraenoic acids [10]. The critical role of leukotriene B_4 suggests that the same mechanism is responsible or at least partially responsible for the antiproliferative effect on lymphoma cells. This notion is supported by the finding that dexamethasone abolished leukotriene B_4 synthesis almost completely [7]. In contrast to control cultures, where leukotriene B_4 was detectable at a constant level within 6 h, no leukotriene B_4 was detectable in dexamethasone-treated cultures. Since addition of leukotriene B_4 to dexamethasone-treated lymphoma cultures failed to reverse the growth inhibition, glucocorticoids appear to induce growth inhibition by more than one mechanism. It must be postulated that glucocorticoids also block the synthesis of other autocrine growth factors and/or the synthesis of growth factor receptors.

D. Glucocorticoid-Induced Lymphoma Cells Lysis

After accumulation of lymphoma cells in G_1 of the cell cycle by interference with the growth regulating system, glucocorticoids initiate cell lysis. The mechanism of corticosteroid-induced cell death has been subject to great controversy. It was proposed that it is the final response to an accumulation of various alterations of cellular metabolism [2]. This hypothesis, however, did not explain the early alterations of chromatin structure after corticosteroid challenge which are characteristic for apoptotic processes and which point to the nucleus having a particular role in the lytic event.

More recently it was postulated by Wyllie [11] that glucocorticoids activate an endonuclease in lymphatic cells, leading to the digestion of internucleosomal DNA and resulting in the discrete distribution of DNA fragments observed following extraction and separation on agarose gel [11]. The characteristic fragment pattern, corresponding to the DNA of oligo- and monosomes, has also been found in S49.1 lymphoma cells [7]. It cannot be ruled out, however, that DNA fragmentation occurs after cell death and merely represents a post-mortem phenomenon. This notion is supported by the finding that a comparable DNA fragment pattern is also detectable in cells treated by other toxic agents such as fluoride, azide, or cycloheximide ([12] and Wielckens, unpublished observations) and is therefore not related to death by necrosis or apoptosis. That a DNA fragmentation process could indeed be crucial for glucocorticoid-induced lymphoma cell lysis can be demonstrated by an indirect approach supporting Wyllie's postulate; however, the mechanism appears to be much more complicated than originally assumed.

The poly(ADP-ribosyl)ation reaction is a nuclear protein modification reaction

utilizing NAD and catalyzed by a chromatin-bound, DNA-dependent enzyme, the poly(ADP-ribose)synthetase [13]. The enzyme polymerizes and transfers ADP-ribose moieties from NAD to chromatin proteins such as histone H2B or topoisomerase I [14, 15]. Moreover, the poly(ADP-ribose)synthetase is extensively automodified [16]. In intact DNA poly(ADP-ribose)synthetase is almost inactive but the enzyme is strongly activated by the introduction of strand breaks [17]. Therefore, it was proposed that the poly(ADP-ribosyl)ation reaction is involved in the DNA repair process [18]. The hypothesis of its involvement in DNA repair is now generally accepted, although the exact role of poly(ADP)-ribosyl)ation during DNA repair has yet to be elucidated.

Since DNA fragmentation either by alkylating agents or irradiation activates the poly(ADP-ribosyl)ation reaction [19, 20], an increase in poly(ADP-ribose)synthetase activity, a rise in the nuclear poly(ADP-ribose) content, and a decrease in the NAD level could reflect DNA fragmentation as well as activation of DNA repair. When S49.1 lymphoma cells were treated with dexamethasone and the poly(ADP-ribose)synthetase activity in permeabilized cells determined at various times after steroid addition, it became obvious that the poly(ADP-ribosyl)ation system was indeed activated during corticosteroid treatment. The amount of active enzyme increased up to fivefold in dexamethasone-treated cells (Fig. 2). By contrast, the amount of active enzyme in control cells was almost stable. The finding of activation of the poly(ADP-ribose)synthetase in dexamethasone-treated lymphoma cells was further supported by analysis of protein-bound poly(ADP-ribose) which revealed a significant increase in the amount of polymeric ADP-ribose residues [21] during the steroid challenge. Moreover, a decline in the NAD level, the substrate of the poly(ADP-ribosyl)ation reaction, was detectable many hours before cell lysis [22] and could be suppressed by addition of an inhibitor of poly(ADP-ribose) formation. The antagonistic effect of poly(ADP-ribosyl)ation in glucocorticoid cell death was also reflected by a strong potentiation of glucocorticoid toxicity by inhibitors of poly(ADP-ribosyl)ation such as benzamide [21, 22].

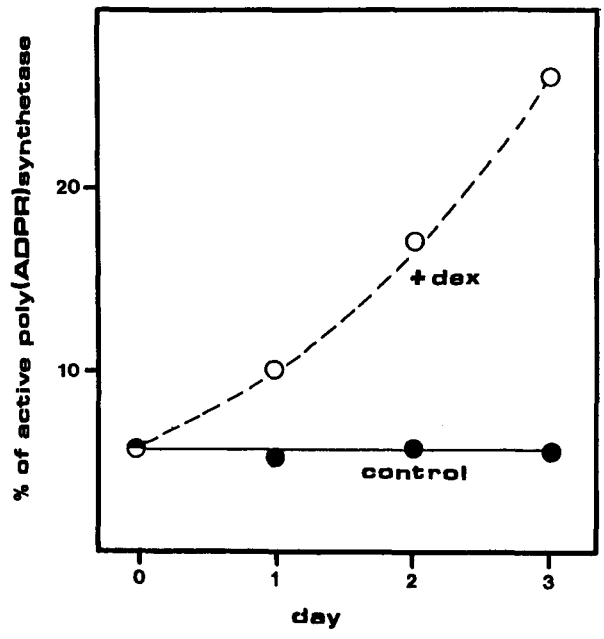

Fig. 2. Activation of poly(ADP-ribose)synthetase in dexamethasone-treated S49.1 lymphoma cells. Cells were incubated in the absence or presence of 10^{-7} *M* dexamethasone (*+dex*) for the times indicated, and the percentage of active poly(ADP-ribose)synthetase was determined as described in [21]

From these data it can be concluded that glucocorticoids really activate an endonuclease, which leads to DNA fragmentation. The activation of DNA repair mechanisms, however, antagonizes the DNA damage as long as sufficient NAD is present. Ultimately, the consumption of NAD limits the ability to repair DNA, and the consequent alterations of DNA repair capacity and breakdown of the energy metabolism together bring about cell lysis (Fig. 3).

Interestingly, benzamide not only enhanced the steroid toxicity but also shifted the initiation of cell death to earlier times. In S49.1 cell cultures treated only with glucocorticoids, cytolysis did not occur until 20–24 h following steroid addition [21]. When dexamethasone and

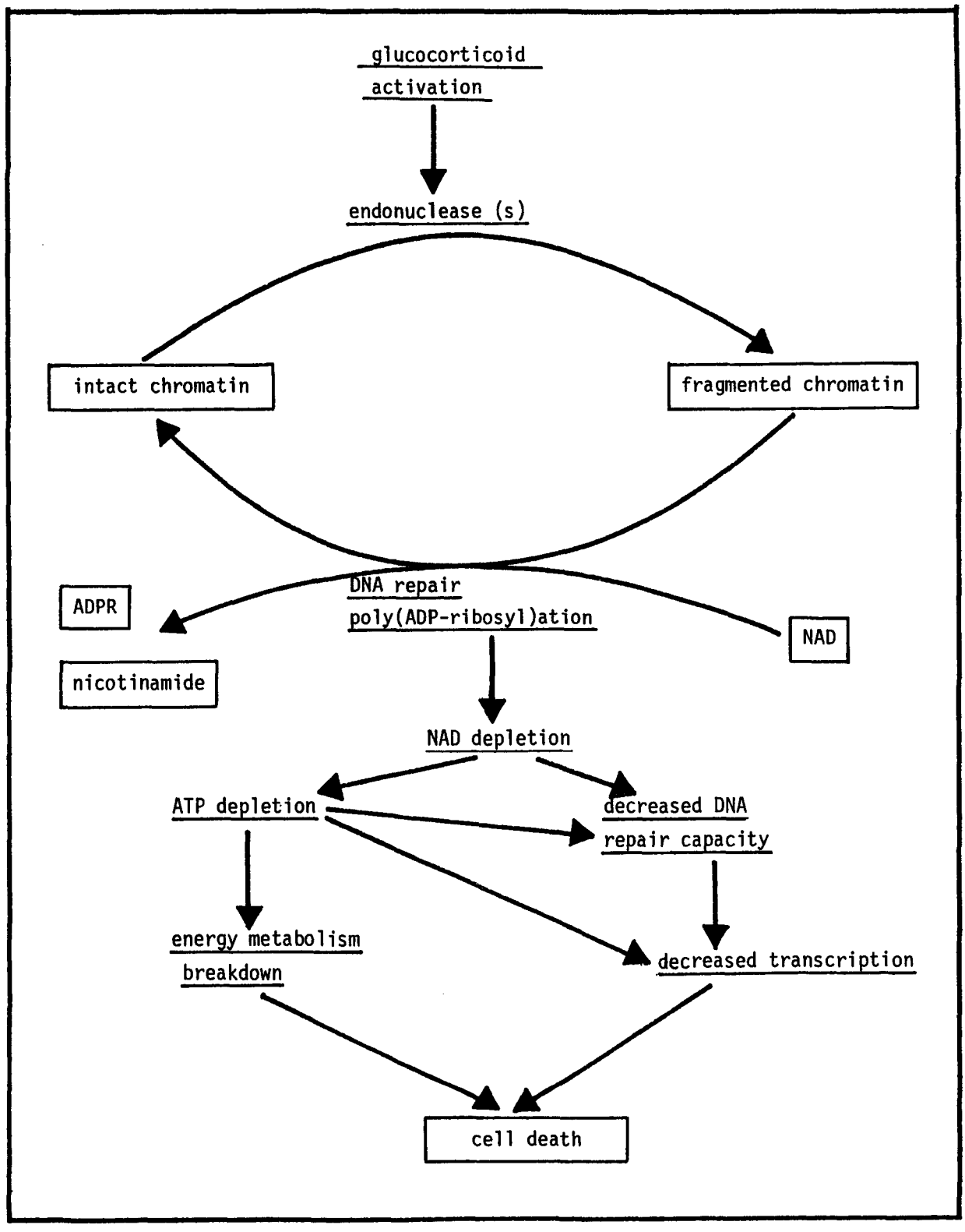

Fig. 3. Possible sequence of phases in response to glucocorticoid challenge

benzamide were combined, however, lysed cells appeared about 10 h earlier [22], a phenomenon easily explained by the idea that DNA repair, including the poly(ADP-ribosyl)ation reaction, antagonizes the glucocorticoid-induced DNA fragmentation. Taken together, the data suggest that three phases in the effect of glucocorticoids on lymphoma cells should be discriminated: a reversible cytostatic phase, a reversible precytolytic phase where endonuclease-mediated

1.	Normal tissue turnover
2.	Embryogenesis
3.	Atrophy of endocrine dependent tissue (e.g., prostate, adrenal cortex)
4.	Treatment with glucocorticoids (lymphocytes, lymphoma cells)
5.	Tumor regression
6.	X-irradiation or radiomimetic cytotoxic agents
7.	T-cell-mediated cell killing
8.	Cold shock
9.	Treatment with tumor necrosis factor
10.	Treatment with lymphotoxin

Table 1. Occurrence of apoptosis

DNA breakage is antagonized by DNA repair, and an irreversible cytolytic phase when the NAD pool is exhausted.

Cellular suicide processes comparable to those during glucocorticoid-induced lymphoma cell death have been demonstrated under a wide variety of conditions (Table 1). Therefore, a cellular suicide system could be essential for the development and homeostasis of a multicellular organism and it is possible that a system similar to that observed in lymphoma cells but triggered by other stimuli is present in nearly every eukaryotic cell. Moreover, the suicide system could be also involved in prevention of malignant transformation.

E. Conclusions

From our data it can be concluded that the therapeutic potential of glucocorticoids in the treatment of malignant lymphomas is not yet fully realized. The effectiveness of steroid therapy could be dramatically increased by combination with agents interfering with the cytostatic or cytolytic potential of the hormone, such as compounds which block the synthesis or binding of growth factors (e.g., inhibitors of the lipoxygenase pathway [7] or suramin [23]) or inhibitors of the poly(ADP-ribosyl)ation reaction, and this may cause no increase in undesirable side effects. Consequently, it is worth while investigating which growth factors are necessary for the proliferation of an individual type of lymphoma or leukemia and developing specific antigrowth factors such as protein growth factor analogs which have no intrinsic activity made by site-directed mutagenesis, antibodies against growth factor receptors, suramin analogs with decreased systemic toxicity, or inhibitors of certain lipoxygenases. The combination of corticosteroids with inhibitors of poly(ADP-ribosyl)ation could also provide a novel approach to the treatment of malignant lymphomas. To date, however, no clinically suitable inhibitor of poly(ADP-ribose)synthetase is available.

References

1. Wyllie AH (1980) Cell death: a new classification separating apoptosis from necrosis. In: Bowen ID, Lockshin RA (eds) Cell death in biology and pathology. Chapman and Hall, London, pp 9–34
2. Munck A, Crabtree GR (1980) Glucocorticoid-induced cell death. In: Bowen ID, Lockshin RA (eds) Cell death in biology and pathology. Chapman and Hall, London, pp 329–359
3. Rosen JM, Fina J, Milholland RJ, Rosen F (1972) Inhibitory effect of cortisol in vitro on 2-deoxyglucose uptake and RNA and protein metabolism in lymphosarcoma P1798. Cancer Res 32:350–355
4. Makman MH, Dvorkin B, White A (1966) Alterations in protein and nucleic acid metabolism of thymocytes produced by adrenal steroids. J Biol Chem 241: 1646–1648
5. Makman MH, Dvorkin B, White A (1968) Influence of cortisol on the utilization of precursors of nucleic acids and

protein by lymphoid cells in vitro. J Biol Chem 243:1485–1497
6. Harmon JM, Norman MR, Fowles BJ, Thompson EB (1979) Dexamethasone induces irreversible arrest and death of a human lymphoid cell line. J Cell Physiol 98:267–278
7. Bittner S, Wielckens K (1988) Glucocorticoid-induced lymphoma cell growth inhibition: the role of leukotriene B_4. Endocrinology 123:991–1000
8. Atluru D, Goodwin JS (1986) Leukotriene B_4 causes proliferation of interleukin 2-dependent T cells in the presence of suboptimal levels of interleukin 2. Cell Immunol 99:444–452
9. Palmberg L, Claesson HE, Thyberg J (1987) Leukotrienes stimulate initiation of DNA synthesis in cultured arterial smooth muscle cells. J Cell Science 88:151–159
10. Davies P, Bailey PJ, Godenberg MM, Ford-Hutchinson AW (1984) The role of arachidonic acid oxygenation products in pain and inflammation. Annu Rev Immunol 2:335–357
11. Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555–556
12. Vedeckis WV, Bradshaw HD (1983) DNA fragmentation in S49 lymphoma cells killed with glucocorticoids and other agents. Molec Cell Endocrinol 30:215–227
13. Ueda K, Hayaishi D (1985) ADP-ribosylation. Annu Rev Biochem 54:79–100
14. Adamietz P, Rudolph A (1984) ADP-ribosylation of nuclear proteins in vivo. J Biol Chem 259:6841–6846
15. Ferro A, Higgins P, Olivera B (1983) Poly(ADP-ribosyl)ation of a topoisomerase. J Biol Chem 258:6000–6003
16. Ogata N, Ueda K, Kawaichi M, Hayaishi O (1981) Poly-(ADP-ribose)synthetase, a main acceptor of poly(ADP-ribose) in isolated nuclei. J Biol Chem 256:4135–4137
17. Miller EG (1975) Stimulation of nuclear (polyadenosine diphosphate ribose) polymerase activity from Hela cells by endonucleases. Biochim Biophys Acta 395:191–200
18. Durkacs BW, Omidiji O, Gray DA, Shall S (1980) (ADP-ribose) participates in DNA excision repair. Nature 283:593–596
19. Wielckens K, Schmidt A, George E, Bredehorst R, Hilz H (1982) DNA fragmentation and NAD depletion. J Biol Chem 257:12872–12877
20. Jacobson EL, Antol KM, Juarez-Salinas H, Jacobson MK (1983) Poly(ADP-ribose) metabolism in ultraviolet irradiated human fibroblasts. J Biol Chem 258:103–107
21. Wielckens K, Delfs T (19869 Glucocorticoid-induced cell death and poly(adenosine diphosphate ribosyl)ation: increased toxicity of dexamethasone on mouse S49.1 lymphoma cells with the poly(ADP-ribosyl)ation inhibitor benzamide. Endocrinology 119:2382–2392
22. Delfs T, Wielckens K (submitted) Glucocorticoid-induced lymphoma cell death and poly(ADP-ribosyl)ation: further investigations on the mechanism of benzamide mediated dexamethasone toxicity increase.
23. Bittner S, Etavard N, Krenz G, Kraus U, Wielckens K (submitted) Suramin potentiation of glucocorticoid toxicity in murine S49.1 lymphoma cells.