Cancer Chemother Pharmacol (1985) 15: 236–239

Cancer Chemotherapy and Pharmacology

Protective effect of testosterone or 5β-dihydrotestosterone pretreatment on CFU-E numbers in busulfan-treated rabbits*, **

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Summary. Busulfan is known to damage hematological stem cells. On the other hand, numerous steroid hormones have a stimulating effect on hematopoiesis. We wished to find out whether steroids have a protective effect on hematopoisis in the case of busulfan treatment. This possibility was tested in vitro in rabbit bone marrow cultures, with and without in vivo pretreatment, with regard to erythropoiesis.

The stimulating effect of testosterone or 5β-dihydrotestosterone on the CFU-E number in rabbit bone marrow cultures was blocked in the presence of busulfan. When rabbits were pretreated with either steroid before the administration of busulfan, however, the reduction of the CFU-E number seen after busulfan therapy alone was compensated.

These results indicate that testosterone and the nonandrogenic 5β-dihydrotestosterone provide some protective effect on erythroid precursor cells when administered with reference to the cell-cycle-dependent action of busulfan.

Introduction

A stimulating effect of numerous steroid hormones on erythropoiesis has been demonstrated by a number of investigators. Beside stimulating renal production of erythropoietin, several steroids exert their effects directly on the bone marrow, and specifically on heme synthesis [13, 14, 19, 25], on erythroid colony-forming cells (CFU-E) [22, 23, 27], and on hematopoietic stem cells [2, 3, 5]. Both androgenic and nonandrogenic steroids are active in this way. On the other hand, the use of most cytostatic drugs involves adverse effects on hematopoiesis. The question arises, therefore, as to whether protection of bone marrow function with steroid hormones is possible during therapy with antineoplastic agents.

Bogliolo et al. [4] studied the alleged possibility of a protective effect of testosterone propionate on hematopoietic cells subjected to the injurious action of several anticancer drugs. These authors were unable to reduce the toxic effect on hematopoietic stem cells in their experimental setting.

* Dedicated to Prof. Dr. Werner Kaufmann, Köln, on the ocassion of his 60th birthday

** Supported by the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, FRG

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However, the mechanism of action of the alkylating agent busulfan differs from that of many other antineoplastic agents insofar as its action with regard to the cell cycle is assumed to be phase-resistant [10]. Busulfan acts at least preferentially on cells in the G_0-phase and not at all or to a much lesser extent on cells in the S-phase of the mitotic cycle [11]. This may explain the very pronounced depressant effect of busulfan on pluripotent hematopoietic stem cells, since most of these cells are not in active cycle [1, 7, 8, 17, 20, 24, 26, 30]. More mature cells, however, are less sensitive or resistant to the action of busulfan. In contrast, when high doses of busulfan were administered to mice, the toxicity was found to be higher in rapidly proliferating spleen colony-forming hematopoietic stem cells (CFU) than is normal bone marrow CFU [31]. Further studies have demonstrated an additional reduction of the proliferating capacity of hematopoietic stem cells surviving busulfan treatment [16]. Furthermore, damage to the microenvironment within the bone marrow has been shown [1, 9, 15, 24].

The target cells in the bone marrow for steroid hormones and for busulfan seem to be identical or closely related. Therefore, it seemed that pretreatment with hormones which trigger these cells into active cycle causing them to undergo self-replication and/or differentiation and maturation, might provide some degree of protection against the damaging effect of subsequent busulfan administration. Thus, the study presented here was undertaken in an attempt to clarify a possible protective effect of steroid hormones on erythropoiesis in busulfan-treated rabbits and to examine its mechanism of action. The potent androgenic compound testosterone (17β-hydroxy-4-androstan-3-one) and the naturally occurring nonandrogenic derivative 5β-dihydrotestosterone (5β-DHT) (17β-dihydroxy-5β-androstan-3-one) were chosen because both steroids are known to have erythropoietic activity in vitro and in vivo [22, 27].

Materials and methods

Details of the methods have been described elsewhere [22]. Briefly, female New Zealand albino rabbits weighing 2.5–3.0 kg were used throughout the experiments. An in vitro erythroid colony-forming system which uses plasma clots according to a modification of the technique described by McLeod et al. [21] was used to determine the number of CFU-E in the bone marrow. Each colony of the

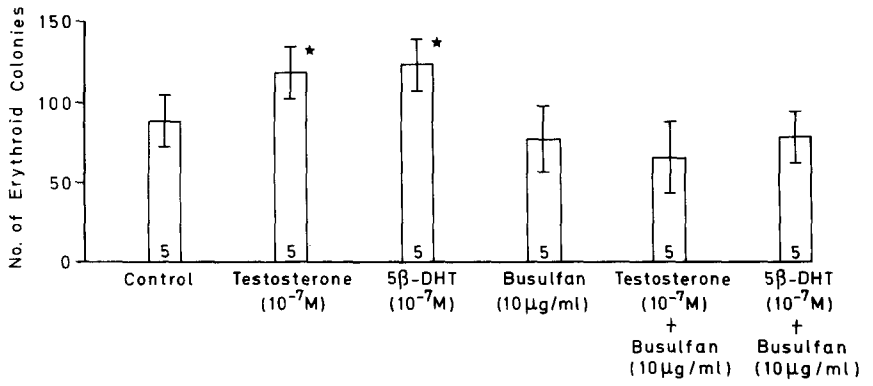

Fig. 1. In vitro erythroid colony formation (CFU-E) per 5×10^4 nucleated bone marrow cells in the presence of testosterone, 5β-dihydrotestosterone, busulfan, testosterone plus busulfan, and 5β-dihydrotestosterone plus busulfan. (mean ± SD). *Significantly different from control ($P < 0.05$). *Number* at the bottom of each *bar* indicates the number of experiments

erythroid type is thought to reflect one precursor cell CFU-E of the bone marrow cell suspension. To test the in vitro effects of the free steroids and/or busulfan, the compounds were added to rabbit bone marrow cultures. Testosterone or 5β-DHT was dissolved in propylene glycol until 5 μl contained the desired amount of the steroid. As previously shown, a molar concentration of 10^{-7} of the steroid was most effective in this culture system [22]. Similarly, busulfan was dissolved in propylene glycol and added to the appropiate cultures so that 5 μl volume contained 10 μg. Equal amounts of the vehicle were present in all cultures shown in Fig. 1. To test the effect of in vivo pretreatment the appropiate steroid (5 mg/kg in propylene glycol) was administered IM 50 h before sacrifice. Control rabbits received the vehicle alone. In the experiments involving busulfan pretreatment this drug was given PO 32 h before sacrifice (20 mg/kg). In the experiments with one of the steroids plus busulfan pretreatment, the steroid therapy preceded busulfan administration by 18 h. The composition of the culture medium, the harvesting procedure for the plasma clots, and the evaluation of the number of the CFU-E were similar to those in our previous studies [22]. The number of erythroid colonies is given per 5×10^4 nucleated bone marrow cells inoculated in 0.1 ml in the wells of microtiter plates. The incubation time of our rabbit bone marrow culture model was 4 days. Four wells were prepared for each determination and the mean number of colonies was calculated. Erythropoietin (0.2 units in 1 ml of the final mixture of the culture medium was necessary for erythroid growth[1]. Five rabbits were used in each set of experiments.

Results

In the presence of either testosterone or 5β-DHT in a molar concentration of 10^{-7} a significant increase in the number of erythroid colonies per 5×10^4 nucleated bone marrow cells was formed in comparison with control cultures ($P < 0.05$) (Fig. 1). Likewise, a significant increase of the CFU-E number was found after pretreatment in vivo with 5 mg/kg of either steroid (Fig. 2). There was no difference from control in the CFU-E number when busulfan was added directly to the cultures (Fig. 1). A significant reduction in the CFU-E, however, was observed in rabbits pretreated with the drug 32 h before they were used for culture ($P < 0.001$ (Fig. 2). Cultures containing both testosterone and busulfan during the incubation period were not different from control, as was correspondingly seen with 5β-DHT (Fig. 1). In contrast, in cultures of cells from rabbits pretreated with either steroid (50 h before sacrifice) plus busulfan (32 h before sacrifice) a significantly higher number of colonies was found than with busulfan therapy alone ($P < 0.005$), but the number was lower than after pretreatment with testosterone or 5β-DHT alone (Fig. 2).

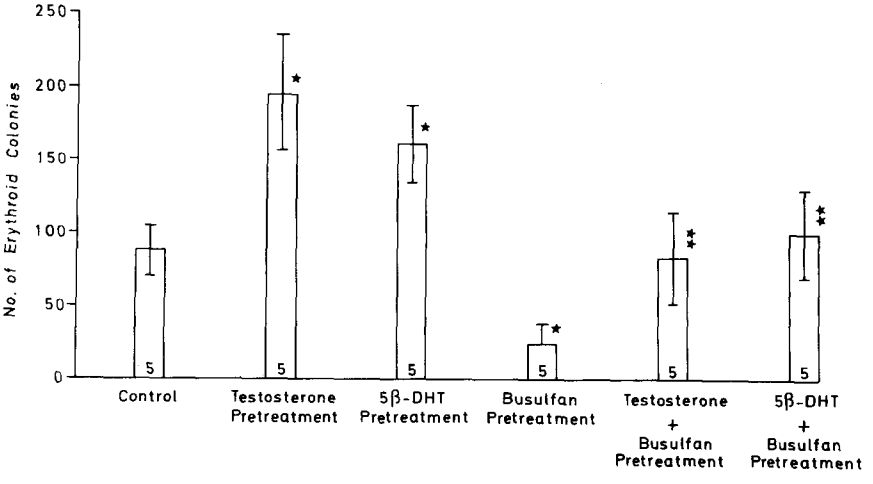

Fig. 2. In vitro erythroid colony formation (CFU-E) per 5×10^4 nucleated bone marrow cells after in vivo pretreatment with testosterone, 5β-dihydrotestosterone, busulfan, testosterone followed by busulfan, and 5β-dihydrotestosterone followed by busulfan (mean ± SD). *Significantly different from control ($P < 0.001$); **significantly different from busulfan pretreatment ($P < 0.005$). *Number* at the bottom of each *bar* indicates the number of experiments

[1] Human urinary erythropoietin with a specific activity of approximately 15 units/mg protein was provided by the National Institutes of Health, Bethesda, Md, USA

Discussion

The androgenic steroid testosterone significantly increased the number of CFU-E detected by eryhtroid colony formation in this culture system, confirming the results of other authors [27]. As has been shown previously, the stimulating effect or testosterone in vitro is completely blocked in the presence of busulfan [22, 23]. Likewise, we have found that the same phenomenon applies to the nonandrogenic, naturally occurring, metabolite 5β-DHT (Fig. 1). Busulfan alone did not change the number of CFU-E when added to the cultures. This indicates that the CFU-E, which is a relatively mature erythroid precursor cell, is not the main site of action of this alkylating agent. These results from our in vitro studies are in agreement with the conclusions of others that busulfan damages stem cells, most of which are not in active cycle but in a resting stage (G_0-phase). More mature cells in active cycle, however, are not affected, or only to a much lesser degree [7, 10, 11, 17].

Testosterone triggers pluripotent hematopoietic stem cells to enter the DNS-phase of the cell cycle [5]. The increase in the number of CFU-E in the presence of testosterone, therefore, could have been due to an influx from a less highly differentiated cell compartment (viz. the stem cells). On the other hand, testosterone could have induced an expansion of the CFU-E compartment by self-replication. In the presence of busulfan, however, which is injurious to stem cells, no testosterone-dependent increase in the CFU-E number was observed (Fig. 1). These results favor the hypothesis that the increase in the number of CFU-E in the presence of testosterone was due to an influx from a more primitive cell compartment rather than to an expansion of the CFU-E compartment upon testosterone-induced self-replication. Since the effects of 5β-DHT applied in vitro were virtually the same as with testosterone, we assume that the target cells for the two steroids are identical or closely related. The mechanism of action of these steroids on erythropoiesis is therefore similar despite the marked difference in the androgenic potency. This aspect may be of importance for possible use of these steroids in humans.

Furthermore, the studies described here demonstrate that pretreatment of rabbits with testosterone or 5β-DHT also increased the numbers of CFU-E in their bone marrow (Fig. 2). In contrast to the in vitro studies, busulfan administration to the rabbits 32 h prior to the preparation of the cell cultures significantly reduced the CFU-E numbers (Fig. 2). This can best be explained by the assumption that over the 32 h in vivo period before culture, the CFU-E compartment was depleted by an ongoing normal flow into the more differentiated erythroid cells. The influx from less highly differentiated cell compartments, however, was reduced or completely stopped by the damaging effect of busulfan on the stem cells.

The question arises as to whether the stem cell compartment can be protected from the effect of busulfan by prior application of steroid hormones. To test this hypothesis testosterone or 5β-DHT was injected IM 18 h before busulfan administration. With this dose and time schedule a significantly higher number of CFU-E was found in the rabbit bone marrow than after treatment with busulfan alone (Fig. 2). This suggests that either of these steroid hormones can act as a protective agent by triggering some of the target cells, viz. stem cells in the G_0-phase of the cell cycle, to DNS synthesis, whereupon they become resistant to the effect of busulfan. These protected cells may then differentiate further and mature or some may undergo self-replication. Further studies are needed to test the size of the stem cell compartment after testosterone or 5β-DHT pretreatment followed by busulfan administration compared with busulfan therapy alone.

While both hormones act directly on the bone marrow (Fig. 1), only testosterone is a potent stimulator of the renal production of erythropoietin, 5β-DHT being inactive in this regard [12]. Therefore, the in vivo effect of testosterone may have been partially mediated by additional erythropoietin production by the kidney. The results of administering 5β-DHT indicate, however, that the protective effect on the CFU-E numbers can also be achieved without stimulation of endogenous erythropoetin production.

Several investigators have demonstrated differences between erythroid and granulocytic differentiation of progenitor cells upon various conditions such as bleeding, phenylhydrazine injection, or busulfan administration [6, 18, 28, 29]. The extent to which the differentiation bias of the hematological pathways is affected by our schedule of steroid plus busulfan treatment has not yet been investigated. Further studies are needed with the granulocytic-macrophage precursor cells (GM-CFC) in a similar culture system. Finally, the effect of variation of time intervals and of prolonged administration on peripheral cell numbers in the blood is not clear.

Our studies do indicate, however, that pretreatment with testosterone or the nonandrogenic metabolite 5β-DHT before therapy with busulfan may have some protective effect on erythroid precursor cells. This is in contrast to the results of other workers who have tested various other cytostatic drugs [4] and is very likely to be related to the peculiar mechanism of action of busulfan on hematopoietic stem cells.

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Received February 4, 1985/Accepted June 4, 1985