

Ectopic Bone Formation after Treatment with Colchicine

Katarzyna TWOREK, Kamila SOLAK, Agnieszka JANECZEK, Tadeusz NIEDŹWIEDZKI,
Zbigniew TABAROWSKI, Zbigniew DĄBROWSKI, André SCHOUTENS, and Stanisław TWOREK

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We followed changes occurring within bone tissue and marrow cells during the process of colchicine-induced ectopic bone development and its resorption inside the marrow cavity of the rat tibia. To stimulate ectopic bone formation male Wistar rats were i.p. injected with 0.5 or 1 mg/kg b.w. of colchicine or with a 100 µg intra-bone injection. Not all subjects responded to colchicine with ectopic bone formation in the marrow cavity, even among individuals belonging to the same strain. The kind of response in a given animal depended on the dose and site of colchicine administration. During 10 days of the experiment an increase in the occurrence of micronuclei in the polychromatic erythrocytes residing in the bone marrow (even 40-fold) was observed, indicating high genotoxicity of colchicine (at a dose of 1 mg/kg b.w. i.p. or 100 µg intra-bone injection). An increase in the frequency of emperipolesis in megakaryocytes between the 4th and 8th days of the experiment was caused by the toxic action of colchicine and may indicate the labilisation of cell membranes and microtubule depolymerisation.

Key words: Bone, ectopic bone, colchicine, emperipolesis, micronucleus.

Katarzyna TWOREK, Kamila SOLAK, Agnieszka JANECZEK, Zbigniew TABAROWSKI, Jagiellonian University, Institute of Zoology, Chair of Animal Physiology, R. Ingardena 6, 30-060 Kraków, Poland.

E-mail: tab@zok.iz.uj.edu.pl

Zbigniew DĄBROWSKI, Jagiellonian University, Institute of Zoology, Chair of Animal Physiology, Ingardena 6, 30-060 Kraków, Poland, and Institute of Physiotherapy, Academy of Physical Education, 31-571 Kraków, Jana Pawła II 78, Poland.

Tadeusz NIEDŹWIEDZKI, Jagiellonian University, Collegium Medicum, Orthopedic and Trauma Clinic, Złotej Jesieni 1, 31-826 Kraków and Institute of Physiotherapy, Academy of Physical Education, Jana Pawła II 78, 31-571 Kraków, Poland.

E-mail: ndzwiedz@mp.pl

André SCHOUTENS, Université Libre de Bruxelles, Clinique Universitaires de Bruxelles, Hôpital Erasme, Service de Médecine Nucléaire, Belgium.

E-mail: ja387867@skynet.be

Stanisław TWOREK, Institute of Nature Conservation, Polish Academy of Sciences, Mickiewicza 33, 31-120 Kraków, Poland.

E-mail: tworek@iop.krakow.pl

Colchicine is an alkaloid isolated from the bulbs of the autumn crocus, *Colchicum autumnale*, and is used as a therapeutic agent in gout. The mechanism of its action consists of blocking the synthesis of lactic acid which leads to an increase in tissue pH from acidic to alkaline. This prevents uric acid crystal formation. Favorable effects of colchicine treatment have also been noted in recurrent polyserositis (familial Mediterranean fever), Behcet disease, sclerodermy, rheumatoid arthritis, osteoarticular inflammation, in the prevention of lung fibrosis after paraquat intoxication, and even in liver cirrhosis (KIMON & GŁADZYSZY 1992). Colchicine is still used in the laboratory to arrest cells at the

metaphase stage of the cell cycle. This enables the study of karyograms with various chromosomal aberrations. It has been found that colchicine acts by blocking (in some cases reversibly) intercellular proteins, mainly tubulin polymerization, which is, among other proteins, a constituent of the cytoskeleton in all eukaryotic cells. Administered colchicine binds with SH groups of tubulin, its B-ring with α tubulin, and A- and C-rings with β tubulin (CHAUDHURI *et al.* 2000) or with carboxyl groups of the tubulin β subunit preventing its polymerization (LILIOM *et al.* 1995). Therefore the dynamic equilibrium between the monomeric and polymeric tubulin forms is disturbed (WOLF &

KNIPLING 1995). Microtubule polymerization is induced by the microtubule initiation fragment, the so-called organizer, cAMP, GTP, Mg^{2+} ions and a factor binding Ca^{2+} ions. Under the influence of colchicine the domination of the monomeric form leads to a reduction in microtubule number in the cytoplasm and cell division is arrested at the mitotic spindle formation stage. Due to these properties colchicine is also used in the clinic as an anti-mitotic drug when treating neoplastic tumors.

The elucidation of the influence of colchicine on cells has focused on tubulin and other proteins of the cytoskeleton that may affect the expression of cell surface molecules. Therefore they influence e.g. the binding of concanavalin A with surface glycoproteins or decrease expression of surface receptors for TNF α , insulin or β adrenergic agonists. Colchicine blocks the formation of the leukotriene \hat{a}_4 complex in the cell membrane and increases the intracellular cAMP level. It also decreases the ability of neutrophils to adhere to endothelial cells by qualitatively changing E-selectin on their surfaces and by the expression of L-selectin on the surface of neutrophils, but not on lymphocytes (CRONSTEIN *et al.* 1995). The influence of colchicine on these factors can in a sense explain its involvement in inflammatory processes in the body. In addition, this drug can induce morphological changes and functional disturbances in cells synthesizing the matrix of the hard tissue such as odontoblasts (OGURA 1967) and osteoblasts (OHYA 1978).

Emperipolesis, first described by HUMBLE *et al.* (1956), is a phenomenon resembling phagocytosis, however, in contrast to phagocytosis, cells engulfed by the cytoplasm of megakaryocytes are kept there without degradation (DEPASQUALE *et al.* 1985; HALIL 1980; LEE 1989). LARSEN (1970) who applied the technique of frame after frame filming showed that various, most often mature blood cells (granulocytes, monocytes, and even erythroblasts) entered and left the cytoplasm of megakaryocytes. Marrow cells after brief contact with megakaryocyte protrusions, pass through their membranes and are surrounded by the cytoplasm, frequently moving on its surface. The system of open canals in the megakaryocyte membrane allows for contact of marrow cells residing within the megakaryocyte cytoplasm with the intercellular matrix. The formation of a fagosome in its classic meaning has never been observed. Megakaryocyte emperipolesis has been shown to increase after administration of some cytokines (STAHL *et al.* 1991).

Small, oval bodies, staining similarly as the nucleus, were observed in the erythrocyte cytoplasm starting from the second half of the XIX century. These bodies were described by HOWELL and

JOLLY (MAVOURNINI *et al.* 1990) and named after their discoverers. SCHMID (1975) and HEDDLE (1973) introduced the micronucleus test to the study of the erythrocyte system in the seventies. In cells with a nucleus, micronuclei are structures usually round or oval in shape, containing chromatin, surrounded by the nuclear membrane but without connections with the nucleus. Micronuclei are easily recognizable within nucleus-lacking mammalian erythrocytes. The very presence and frequency of micronuclei under the influence of various toxic agents is usually estimated in the polychromatic erythrocyte PCE (still having ribosomal RNA in their cytoplasm). The erythrocyte micronucleus test is applied in studies using experimental animals, mainly mice and rats. The number of polychromatic erythrocytes with a micronucleus (MNPCE) is most often analyzed in the bone marrow and peripheral blood cells and spleen replicas as described by HAMADA *et al.* (2001). Micronuclei are biological indicators of genotoxicity. In view of the studies on apoptosis, it is believed that cells having a micronucleus (micronuclei), die by a process of apoptosis called "delayed apoptosis". This occurs after exposition to small doses of toxic agents causing irreversible damage. However, the possession of a nucleus permits the completion of at least one cell cycle.

Bone development on both endochondral and membranous substratum is a physiological process. Bone development in atypical locations (ectopic) is another issue. This process may occur in two ways:

- (1). it can be caused by poorly defined agents on a genetic background (e.g. osteopetrosis) or postinjury (e.g. in the muscles of paraplegics);
- (2). it may be provoked by various substances, most often through experimental procedures.

Interesting, but requiring further research, are the results of experiments using colchicine and vinblastin, alkaloids called microtubular "poisons" causing ectopic bone formation inside the marrow cavity. Such studies are carried out in two centers: in the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Tokyo Medical and Dental University and in the Centro Italiano Ricerca e Produzione di Farmacie di Biotecnologia da Ingegneria Genetica in un Polo Tecnologico Europeo Avanzato, L'Aquila.

The aim of this study was to describe the influence of colchicine on the process of ectopic bone formation within the marrow cavity and its subsequent resorption by means of the follow up of the bone tissue volume and marrow cells number.

Material and Methods

Animals

The experiment was carried out on 208 sexually mature Wistar male rats, weighing 190 to 260 grams (223.7 ± 19.6 , mean \pm SD). Animals were kept in controlled conditions L/D – 12/12, fed with LSK chow and water *ad libitum*. The experiments were approved by the local Ethical Committee of the Jagiellonian University.

Animals were weighed before injections with colchicine (experimental groups) or physiological saline (control series) and just before killing. Experimental rats were divided into three groups depending on the injection site and the dose of colchicine while control animals were placed into two groups depending on injection site.

I E group. Animals from this group received 100 μ g of colchicine per kg bw in 0.05 ml of physiological saline injected directly into the tibial marrow cavity. Rats were killed by a lethal dose of vetbutal (0.5-0.7 ml depending on their weight) 3, 4, 5, 6, 7, 8, 9 or 10 days after injection. 6 animals were studied for each post-injection day.

Surgery was performed under general anesthesia using a vetbutal volume appropriate for each animal mass from 0.2 to 0.4 ml. A small incision 4-5 mm long was made above the tibia 5-7 mm from the knee joint. Then muscles were separated by layers, the periosteum incised, the bone exposed and a 1 mm diameter hole was drilled into the bone cortex, through which colchicine was injected. In order to minimize colchicine leakage, the injection was performed slowly over 90 seconds. The hole was sealed with surgical wax, the periosteum stitched with two catgut 00 stitches, while the skin incision was clipped with 3-4 clamps. The operation site was sprayed with Acutol. No post surgery complications were observed.

I C group. Control animals received injections of 0.05 ml of physiological saline into their marrow cavities. All experimental procedures and surgery were strictly the same as previously described for group I E, with the only difference that the number of sacrificed animals was 4 for each post-injection day.

II E group. Rats from this group received intraperitoneal (i.p.) injections of colchicine (1 mg per kg bw in 0.25 ml of physiological saline) and were sacrificed by a lethal dose of vetbutal 3, 4, 5, 6, 7, 8, 9 or 10 days after injection. There were 6 animals studied per each post-injection day.

III E group. Rats from this group received i.p. colchicine injections (0.5 mg per kg bw in 0.25 ml

of physiological saline) and were sacrificed by a lethal dose of vetbutal 3, 4, 5, 6, 7, 8, 9 or 10 days after injection. There were 6 animals sacrificed per each day.

II/III C groups. Control animals received i.p. injections of 0.25 ml of physiological saline. Four animals were sacrificed with an overdose of vetbutal, 3, 4, 5, 6, 7, 8, 9 or 10 post-injection day.

Histological and cytological methods

After killing the tibias were excised, cleaned from the surrounding muscles and periosteum and X-rayed. A small piece of marrow was taken out from the epiphyseal part of the bone and a few smears were made. Then the bone was placed in Schaffer fixative. After fixation and dehydration in a series of alcohol the bone was embedded in methacrylate resin according to ISLAM and FRISCH (1985). Bones were cut longitudinally (left tibia) and transversely (right tibia) on a microtome. The 3-5 μ m thick sections were mounted and stained with the Goldner trichrome method, hematoxylin and eosin, and with van Kossa staining in order to study the level of mineralization and osteone growth. Marrow smears and sections were stained for the activity of alkaline phosphatase according to RUTENBURG *et al.* (1965), and for the activity of acid phosphatase according to LI *et al.* (1970). Marrow smears were stained with routine methods: May-Grünwald – Giemsa (using Serva stains and procedures) and with Giemsa – Wright. Megakaryocyte emperipolexis was estimated in 25 to 50 cells per each animal. A quantitative micronucleus test in polychromatic erythrocytes (MNPCE) was performed on 1000 erythrocytes (in %). The area of the newly formed bone in the marrow cavity was estimated from histological preparations according to SATO and BYERS (1994) – as modified by our statistics specialist dr Jack Domosławski. This modification was based on the Tissue Analysis System (TAS), with TV camera and a computer. The expansion of bone tissue in the marrow cavity was examined over an area of 3200 μ m \times 2300 μ m in 5-10 randomly chosen sites of each preparation. These preparations were obtained from the tibia cut transversely at a 5-7 mm distance depending on the animal weight from the horizontal articular surface of the tibia epiphysis.

Calculations were based on an equation in which the estimated increase of bone volume in the marrow cavity (BV – bone volumetry) was the sum of the area of trabecular spongy bone N_B , and of the osteoid component – N_O , divided by the sum of $N_B + N_O +$ bone marrow N_M .

$$BV = \left\{ \left(\sum N_B + \sum N_O \right) / \left(\sum N_B + \sum N_O + \sum N_M \right) \right\} \times 100 (\%)$$

Measurements of the osteoid tissue (N_O) were performed using a polarization microscope on van Kossa stained sections.

Results

Ectopic bone increase in the marrow cavity

Between days 5 and 6 after injection of colchicine, the responding animals from group I E (Table 1 & 2, Fig. 1) exhibited a hypocellular marrow surrounding well formed sinuses (*sinus venosus*) filled with blood. Both in the bone and on the margins of the marrow lacunae numerous osteoblasts were present. In the first post-injection days (1-4), marrow was characterized by a high activity of alkaline phosphatase within neutrophils and osteoblasts (Fig. 2), while in the final days of the experiment there was an increased number of osteoclasts showing an intense reaction for acid phosphatase (Fig. 3). The animals from group II E received an i.p injection of colchicine. The formation of ectopic bone inside the marrow cavity was 33% slower when compared with animals from group I E.

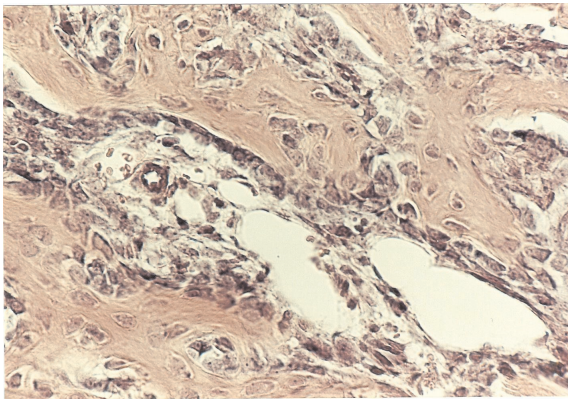


Fig. 1. Bone tissue formed ectopically within the marrow cavity (arrows) of a rat tibia 6 days after colchicine injection. In places without bone, venous sinuses surrounded by hypocellular marrow can be seen. Rat from the E I group. $\times 400$, H&E.

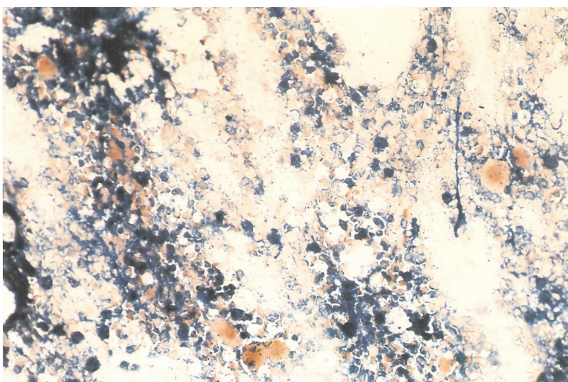


Fig. 2. Marrow smear from a group I E rat made 5 days after colchicine injection. High activity of alkaline phosphatase in osteoblasts and neutrophils (arrows). Positive histochemical reaction indicated by navy blue color. $\times 400$.

Emperipolesis

The phenomenon of intensified emperipolesis was present starting from day 4 after colchicine injection in groups I E and II E. At days 4 and 5 emperipolesis was detected in 44% and 45% of megakaryocytes in both groups. The percentage of affected cells was higher on days 7 and 8. No observations could be made on the 9th and 10th days, since bone marrow cellularity was quite poor and no megakaryocytes were available. The cells most often encompassed by megakaryocytes were polymorphonuclear granulocytes (Table 3, Fig. 4) In groups III E, I C and II/III C emperipolesis was detected at a stable level of 12% (± 2).

Micronucleus test

There was a significant increase in the number of polychromatic erythrocytes with micronuclei in rats from experimental groups IE and IIE between days 3 and 10 (Table 4). From the 3rd day after injection of colchicine both into the tibial marrow cavity and intraperitoneally, a gradual increase in

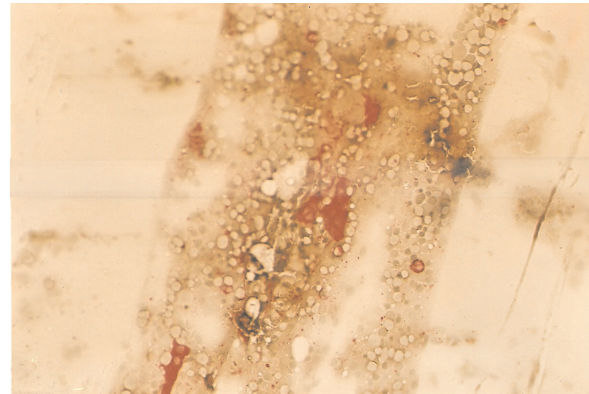


Fig. 3. Marrow smears made from a rat from group II E 10 days after colchicine injection. High activity of acid phosphatase in osteoclasts (arrows) indicated histochemically by red color. $\times 400$.

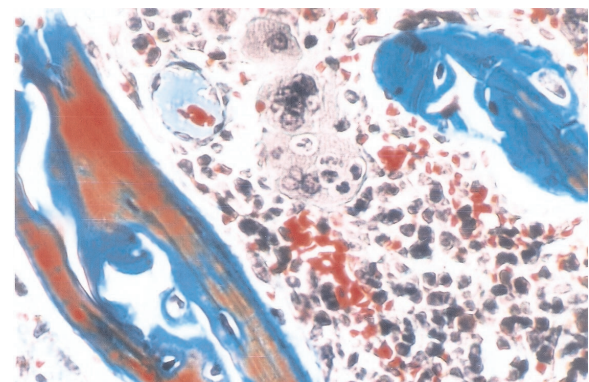


Fig. 4. A megakaryocyte with clearly enhanced emperipoletic activity. From a rat from group I E, 3 days after colchicine injection. Around the nucleus in the megakaryocytes, cytoplasm engulfed blood cells (arrows) in various developmental stages can be seen. Giemsa-Wright, $\times 1000$.

the number of cells with micronuclei from 17% at day 3 up to 43.2% at day 6 was observed. A slight decrease in the number of polychromatic erythrocytes with micronuclei was noted between days 7 and 10 of the experiment. Intraperitoneal administration of 0.5 mg of colchicine per kg/body weight in 0.25 ml of physiological saline caused a fractional increase in the number of polychromatic erythrocytes with micronuclei when compared with control groups (IC and II/III C).

Discussion

Colchicine and ectopic bone formation

A short report on bone formation in the marrow cavity of Wistar male rats following injection of colchicine, with simultaneous progressive necrosis of marrow cells and degeneration of marrow vasculature, was published for the first time in 1980 (OGURA *et al.*). In the 9th-10th day after colchicine injection these authors noted a return to the initial status and an increase in hematocrit value. The same effect was also obtained by TAMURA (1986) in male Wistar rats by an intravenous injection of 2.5 μ mol of colchicine per kg b. w., and a weaker effect after using vinblastine. This author performed a detailed histological analysis of the bone formed within the marrow cavity. This study was carried out on 125 rats. However, in both discussed papers there was no information indicating what percentage of animals actually responded to colchicine with ectopic bone formation within the marrow cavity. Some technical details employed in our study such as the dose of colchicine (1 mg/kg b.w. – group II E), the measured area of surface of the bone formed within the marrow cavity ($3.2 \times 2.3 \text{ mm}^2$) and number of times when rats were sacrificed after the injection of colchicine are similar to those employed by TAMURA. New aspects in our study include the micronucleus test on polychromatophile erythrocytes, as well as megakaryocyte emperipolesis discussed in the following sections. The experiment was performed on rats belonging to the Wistar strain, however a substantial number of substrains has been described within this strain by FESTING and STAATS (1973). This may not result in new bone formation in all animals even though colchicine was applied directly into the marrow cavity. This method of colchicine administration was most efficient because in this group (I E) the highest percentage of animals responded by bone formation within the marrow cavity. With the exception of the paper by WŁODARSKI and WŁODARSKI (1997), other authors did not provide information on the per-

centage of animals responding to colchicine with new bone formation within the marrow cavity. This may suggest that all rats after a single i.v. dose of 1 mg/kg b. w. of colchicine responded with bone formation within the marrow cavity. However, in our experiment only 43.75% of rats receiving colchicine directly into the marrow cavity responded by ectopic bone formation. This value in rats receiving colchicine i.p. at a dose of 1 mg/kg b.w. was 29.16%, whereas at a dose of 0.5 mg/kg b.w. only 2% of experimental animals responded by ectopic bone formation. WŁODARSKI and WŁODARSKI (1997) injected 0.04 mg of colchicine into the tail vein of 24 mice of both sexes of BALB/c and CFW/L1 strains, and 0.2 mg of colchicine to 6 male WAG strain rats weighing 150 g. They studied the efficacy of injected colchicine with the mitotic index of ear epidermal cells. Mice were killed 2, 6, 7, 8, 10, 11, 15 and 26 days after injection of colchicine. They did not find ectopic bone formation in the studied rodents. Only on post-injection days 6 and 7 did they note necrotic changes in the bone marrow with preservation of the sinuses. Rats killed on days 4, 9 and 12 after injection did not exhibit such changes. These authors criticized results obtained by ADACHI (1987) and ARAI *et al.* (1993), ascribing changes in the marrow cavity to randomly spotted osteopetrosis, which rarely occurs in rodents due to a genetic defect. In a letter to the editor of the journal, CASTELLI *et al.* (1999) challenged the opinion expressed by WŁODARSKI and WŁODARSKI citing results of their studies which are in agreement with ours. It should be noted that the absence of ectopic bone formation within the marrow cavity after injection of colchicine in mice and rats obtained by WŁODARSKI and WŁODARSKI is in accordance with results from our group III E. These animals received 0.5 mg of colchicine i.p. In this group only one rat responded with an increased number of bone trabeculae on the 6th day after colchicine injection. In the remaining groups (I E and II E), with 48 animals each, the percentage of animals that responded with ectopic bone formation within the marrow cavity amounted to 43.75% and 29.16%, respectively. The differences between results obtained by WŁODARSKI and WŁODARSKI and our study can be ascribed to different rat strains used in the experiment and different observation days. In our experiment the most pronounced changes in the marrow cavity were noted between days 6 (group III E) and 8 (groups I E and II E). OHYA (1999 – personal communication) shares this opinion.

The osteogenic effect of colchicine obtained in our study is in contrast to that described by BASTOW *et al.* (1993) who studied the effect of colchicine and its two derivatives on human osteo-

genic sarcoma cells *in vitro*. This phenomenon can be attributed to the different affinity of neoplastic cell membranes to cytostatics than those of normal cells, and a different experimental setting, i.e. *in vivo*. Administered colchicine undergoes internal biodegradation to various degrees (HUNTER & KLAASSEN 1975). In experiments carried out on rats it has been found that 68% of a single dose of colchicine is excreted within 48 hours with bile mainly as desmethylcolchicine. These authors have also noted high individual differences in its excretion which may account for the formation or absence of ectopic bone within the marrow cavity observed in our study (Table 1). We still have too little information concerning the mechanisms of colchicine action, but radioimmunological methods (POULEV *et al.* 1994; SABOURAUD *et al.* 1994) allow for precise localization of colchicine in tissues and body fluids. This supports the results of a study by WALASZEK *et al.* (1960) in which labeled colchicine was applied. These authors found that 20 minutes after administration of colchicine (2 µg/kg bw) its highest concentration was in the bile, liver, kidney and spleen, a slightly lower concentration in lungs and muscles, and the lowest in neural tissue (levels in marrow and bone were not studied). These investigations have shown differences in the distribution of colchicine in particular organs which may explain the irreproducibility of its effects.

Bone marrow cells injected intramuscularly (DUDKIEWICZ *et al.* 2005) or into the spleen (DĄBROWSKI 1975) greatly increase the number of osteogenic cells, leading to heterotrophic ossification. In the experiment by DUDKIEWICZ *et al.* (2005), in which 0.25 mg of colchicine was administered daily to 12 rabbits via a feeding tube combined with the administration of bone marrow cells, ectopic bone formation was detected in 3 individuals. However, the authors did not examine the interior of the medullary cavity for ectopic bone formation. In our opinion the accumulation of trabecules is scarcely visible on X-ray figures of animals that received colchicine.

Colchicine and emperipolesis

In physiological conditions in our experiment emperipolesis was detected in 10-14% of megakaryocytes (Table 3, Fig. 4), while after injection of colchicine its frequency increased up to 52%. Obviously this concerned sites in which marrow hematopoietic islets were present between days 4 and 8 of ectopic bone formation. Together with the increase of the volume of newly formed ectopic bone, marrow cells were displaced by megakaryocytes. The increased incidence of emperipolesis has been described in cases of myeloproliferative

diseases, Hodgkin disease, multiple myeloma, neuroblastoma, AML and CML and in hemolytic anemia (SHAMOTO 1981; SOBOLEWSKI 1980). A subtle dose of ionizing radiation also increased megakaryocyte emperipolesis (BOBIK & DĄBROWSKI 1995). In our experiment colchicine, a microtubular toxin, clearly caused an increase in emperipolesis. In the literature (BURKHARD *et al.* 1984) there were attempts to establish emperipolesis as a diagnostic feature in toxicity tests or in the diagnosis of onco-hematological diseases of myeloproliferative character. BAATOUT *et al.* (1998, 1998) have shown that megakaryocytes are very sensitive to colchicine. These cells responded with an increase in the ploidy of cell nuclei, as well as with the appearance of nucleoli within nuclei. This was accompanied by an increased protein synthesis from 200 to 300 per DNA unit. The mitotic index also increased within these limits. On the basis of changes occurring within megakaryocytes after incubation with colchicine, it can be stated that they are highly susceptible (almost selectively) to this compound. With such intense changes within the cytoplasm of megakaryocytes, most probably there is some labilisation of the cell membrane reflected by an increased incidence of emperipolesis observed in our studies. Papers concerning the effect of colchicine on megakaryocytes are still scarce (VAN-DER-LOO *et al.* 1993; BAATOUT *et al.* 1998). TAVASSOLI (1981, 1986) devoted an important part of his studies to megakaryocyte emperipolesis. He emphasized the connection between blood loss and the level of emperipolesis. He also defined an emperipoletic index as the frequency of megakaryocytes (their number) times the number of cells present within their cytoplasm. Moreover he described a decrease in this index after acute blood loss and its increase after chronic blood letting in animals. The phenomenon of emperipolesis observed in our studies can be explained using the concept proposed by TAVASSOLI and AOKI (1989), referring to megakaryocytes as constitutive elements of marrow sinuses (apart from endothelial, adventitial cells and the basement membrane). Mature blood cells must pass from the marrow parenchyma into the peripheral blood through their bodies, i.e. trans-megakaryocytally. The increase in this phenomenon observed between days 4 and 8 of our experiment can be explained by "salvage stimulation" of proliferating marrow cells being displaced and squeezed by increasing bone tissue growing within the marrow cavity.

Colchicine and the micronucleus test

In 1986 SATYA-PRAKASH *et al.* described damage to the chromosomes of mice marrow cells after

colchicine administration. GUDI *et al.* (1992) have observed a 55% increase in the number of micronuclei in polychromatic erythrocytes (MNPCE) 24 hours after colchicine administration at a dose of 1 mg/kg b.w. In our experiment an increase in the frequency of micronuclei in polychromatophilic erythrocytes was detected until the 10th day of the experiment. A group of researchers associated in the organization Collaborative Study Group for the Micronucleus Test (CSGMT 1992) gathered data concerning the frequency of micronuclei induced in PCE in mice treated with various toxic (including colchicine), and non-toxic compounds. They have shown a significant effect of colchicine on the formation of micronuclei in PCE. Acridin orange staining was applied as described by HAYASHI *et al.* (1992). HAYASHI *et al.* (1983) highly value the usefulness of the micronucleus test, especially when testing genotoxic substances. According to these authors the application of this test is justified using both simple light microscopy evaluation (with Giemsa staining) or with acridine orange and flow cytometry. Finally SCHRIEVER-SCHWEMMER *et al.* (1997) in their studies on the influence of colchicine on the number of micronuclei in polychromatophilic erythrocytes from mice marrow applied a "paintbrush" method. The conclusions of the studies mentioned above are in line with our results in which we also observed a substantial increase in the number of micronuclei in PCE (this concerned mainly groups I E and II E). The higher number of such erythrocytes remained until the 10th day of the experiment. We noted an increase in number of micronuclei among cells of regenerating marrow within hematopoietic "islets" located in retreating ectopic bone tissue in the marrow cavity. In animals which did not respond with the formation of the ectopic bone in the marrow cavity it was easy to count micronuclei in marrow smears. The increase in the number of micronuclei in PCE was an important test proving their reaction to the administered colchicine, especially in those animals which did not respond with ectopic bone formation in the marrow cavity. The results of the micronucleus test for control animals were stable at the level of $1.1\% \pm 0.3$, which is in line with results published by HAMADA *et al.* (2001), who studied 14 rat strains.

There is still a lot of controversy around the origin of ectopic bone formation after colchicine injection. Some authors claim that this does not occur (WŁODARSKI & WŁODARSKI 1997) while others (CASELLI *et al.* 1999) observed this phenomenon in the majority of animals tested. In our experimental setting one group of studied animals, i.e. those receiving colchicine directly into the

marrow cavity, developed bone structures within the marrow cavity. However, in prospective investigations more strains of mice, rats and rabbits should be tested both *in vivo* and *in vitro* to elucidate the possible genetic background of differences in responsiveness to colchicine.

References

- ADACHI M. 1987. Characterization of osteoblast precursor cells isolated from colchicine treated rats. *Bull. Tokyo. Med. Dent. Univ.* **34**: 41-51.
- ARAI N., OHYA K., OURA H. 1993. Osteopontin mRNA expression during bone resorption: an *in situ* hybridization study of induced ectopic bone in the rat. *Bone Miner.* **22**: 129-145.
- BAATOUT S., CHATELAIN B., STAQUET P., SYMANN M., CHATALEAIN C. 1998. Inhibition of tubulin polymerization in megakaryocyte cell lines leads to polyploidization which affects the metabolism of actin. *Anticancer Res.* **18**: 1553-1561.
- BAATOUT S., CHATELAIN B., STAQUET P., SYMANN M., CHATELAIN C. 1998. Augmentation of the number of nucleolar organizer regions in human megakaryocyte cell lines after induction of polyploidization by a microtubule inhibitor. *Eur. J. Clin. Invest.* **28**: 138-144.
- BASTOW K. F., TATEMATSU H., BORI I. D., FUKUSHIMA Y., SUN L., GOR B., LEE K. 1993. Induction of reversible protein-linked DNA breaks in human osteogenic sarcoma cells by novel cytotoxic colchicine derivatives which inhibit DNA topoisomerase II *in vitro*: absence of cross-resistance in a colchicine-resistant sub-clone. *Bioorg. Med. Chem. Let.* **13**: 1045-1050.
- BOBIK R., DĄBROWSKI Z. 1995. Emperipolesis of marrow cells within megakaryocytes in the bone marrow of sublethally irradiated mice. *Ann. Hematol.* **70**: 91-95.
- BURKHARDT R., KLEINKNECHT R., JAGGIER R., FRISH B., MAHL G., BARTL R. 1984. Megakaryocytic emperipolesis – accidental or diagnostic sign? (In: Pathology of the Bone Marrow. Lenner K., Hubner K. eds. G. Fischer, Stuttgart): 200-205.
- CASELLI G. F., FIORENTINO S., RIMINUCCI M., CORSI A., BIANCO P. 1999. Does colchicine really induce bone formation in the rodent bone marrow? Yes, it does. *Calcif. Tissue Int.* **65**: 414-415.
- CHAUDHURI A. R., SEETHARAMALU P., SCHWARZ P. M., HAUSHEER F. H., LUDUENA R. F. 2000. The interaction of the B-ring of colchicine with α -tubulin: A novel footprinting approach. *J. Mol. Biol.* **303**: 679-692.
- CRONSTEIN B. N., MOLAD Y., REIBMAN J., BALAKHANE E., LEVIN R. I., WEISSMAN G. 1995. Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. *J. Clin. Invest.* **96**: 994-1002.
- CSGMT (The Collaborative Study Group for the Micronucleus Test) 1992. Micronucleus test with mouse peripheral blood erythrocytes by acridine orange supravital staining: The summary report of the 5th collaborative study by CSGMT/JEMSMMS. *Mutat. Res.* **278**: 83-98.
- DĄBROWSKI Z. 1975. Autotransplantations of spleen fragments into bone marrow and bone marrow into the spleen of rabbits. *Zesz. Nauk. Univ. Jagiell. Pr. Zool.* **393**: 105-178.
- DEPASQUALE A., PATERLINI P., QUAGLINO D., QUAGLINO D. 1985. Emperipolesis of granulocytes within megakaryocytes. *Br. J. Haematol.* **60**: 384-386.
- DUDKIEWICZ I., COHEN I., HOROWITZ S., REGEV S., PERELMAN M., CHECHIK A., LANGEVITZ P., STRASBURG S., LIVNEH A., SALAI M. 2005. Colchicine inhibits heterotopic ossification: Experimental study in rabbits. *Isr. Med. Assoc. J.* **7**: 31-34.

- FESTING M., STAATS J. 1973. Standardized nomenclature for inbred strains of rats. *Transpl.* **16**: 221-229.
- GUDI R., XU J., THILAGAR A. 1992. Assessment of the *in vivo* aneuploidy/micronucleus assay in mouse bone marrow cells with 16 chemicals. *Environ. Mol. Mutagen.* **20**: 106-116.
- HALIL O. 1980. Phagocytosis by megakaryocytes in malignant disorders. *Br. J. Haematol.* **46**: 161-165.
- HAMADA S., YAMASAKI K., NAKANISHI S., OMORI T., SERIKAWA T., HAYASHI M. 2001. Evaluation of the general suitability of the rat for the micronucleus assay: the effect of cyclophosphamide in 14 strains. *Mutat. Res.* **495**: 127-134.
- HAYASHI M., NORRPA H., SOFUNI T., ISHIDATE M. Jr. 1992. Mouse bone marrow micronucleus test using flow cytometry. *Mutagenesis* **7**: 251-256.
- HAYASHI M., SOFUNI T., ISHIDATE M. Jr. 1983. An application of acridine orange fluorescent staining to the micronucleus test. *Mutat. Res.* **120**: 241-247.
- HEDDLE J. A. 1973. A rapid *in vivo* test for chromosomal damage. *Mutat. Res.* **18**: 87-190.
- HUMBLE J. G. G., JAYNE W. H. W., PULVERTAFT R. J. V. 1956. Biological interaction between lymphocytes and other cells. *Br. J. Haematol.* **2**: 283-294.
- HUNTER A. L., KLAASSEN C. D. 1975. Biliary excretion of colchicine. *J. Pharmacol. Exp. Ther.* **192**: 605-617.
- ISLAM A., FRISCH B. 1985. Sections of undecalcified marrow cores. *Histopathol.* **9**: 1263-1274.
- KIMON K., GLĄDZYSZ A. 1992. Colchicine – mechanism of action and clinical trials of its use in hepatology. *Przeg. Lek.* **49**:234-236. (In Polish).
- LARSEN T. E. 1970. Emperipolesis of granular leukocytes within megakaryocytes in human hemopoietic bone marrow. *Am. J. Clin. Pathol.* **53**: 485-489.
- LEE K. P. 1989. Emperipolesis of hematopoietic cells within megakaryocytes in bone marrow of the rat. *Vet. Pathol.* **26**: 473-478.
- LI C. Y., YAM L. T., LAM K. W. 1970. Acid phosphatase isoenzyme in human leukocytes in normal and pathological conditions. *J. Histochem. Cytochem.* **8**: 473-479.
- LILJOM K., LEHOTZKI A., MOLNÁR A., OVÁDI J. 1995. Characterization of tubulin-alkaloid interactions by enzyme-linked immunosorbent assay. *Anal. Biochem.* **228**: 18-26.
- MAVOURINI K. H., BLAKEY D. H., CIMINO M. C., SALAMONE M. F., HEDDLE J. A. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene – Tox. Program. *Mutat. Res.* **239**: 29-80.
- OGURA H. 1967. Study of the inhibitory action of colchicine on dentin formation. *Jpn. J. Oral Biol.* **9**: 8-12
- OGURA H., SENZAKI H., OHYA K. 1980. Overproduction of bone in the marrow of rat tibia after colchicine administration. *Jpn. J. Pharm.* **30** Suppl.: 235.
- OHYA K. 1978. Effects of colchicine on osteoblast in rat: An ultrastructural study. *Bull. Tokyo Med. Dent. Univ.* **25**: 277-295.
- POULEV A., DEUS-NEUMANN B., BOMBARDELLI E., ZENK M. H. 1994. Immunoassays for the quantitative determination of colchicine. *Planta Med.* **60**: 77-83.
- RUTENBURG A. M., ROSALTES C. L., BENNETT J. M. 1994. An improved histochemical method for the demonstration of leukocyte alkaline phosphatase activity in clinical application. *J. Lab. Clin. Med.* **65**: 698-704.
- SABOURAUD A., CANO N., SCHERMANN J. M. 1994. Radioimmunoassays of colchicine with antisera exhibiting variable cross-reactivity. *Ther. Drug Monit.* **16**:179-185.
- SATO K., BYERS P. D. 1994. Histomorphometric study of intratrabecular osteons in the iliac bone in three metabolic bone diseases. *Tohoku J. Exp. Med.* **172**: 317-326.
- SATYA-PRAKASH K. L., LIANG J. C., HSU T. C., JOHNSTON D. A. 1986. Chromosome aberration in bone marrow cells following treatment *in vivo* with vinblastine and colcemid. *Environ. Mutagen.* **8**: 273-282.
- SCHMID W. 1975. The micronucleus test. *Mutat. Res.* **31**: 9-15.
- SCHRIEVER-SCHWEMMER G., KLLIESCG U., ADLER I. D. 1997. Extruded micronuclei induced by colchicine or acrylamide contain mostly lagging chromosomes identified in paintbrush smears by minor and major mouse DNA probes. *Mutagenesis* **12**: 201-207.
- SHAMOTO M. 1981. Emperipolesis of hematopoietic cells in myelocytic leukemia. *Virchows Arch.* **35**: 283-290.
- SOBOLEWSKI S. 1980. Phagocytosis by megakaryocytes in malignant disorders (abstract). *Br. J. Haematol.* **45**:173.
- STAHL C. P., ZUCKER-FRANKLIN D., EVATT B. L., WINTON E. F. 1991. Effects of human interleukin-6 on megakaryocytes development and thrombopoiesis in primates. *Blood* **78**: 1467-1475.
- TAMURA Y. 1986. Study on ectopic calcification in bone marrow of rat tibia caused by vinblastine. *Bull. Tokyo Med. Dent. Univ.* **33**: 9-22.
- TAVASSOLI M. 1981. Emperipolesis by megakaryocytes in blood loss. *Br. J. Haematol.* **49**: 660-661.
- TAVASSOLI M. 1986. Modulation of megakaryocyte emperipolesis by phlebotomy: megakaryocytes as a component of marrow-blood barrier. *Blood Cells* **12**: 205-216.
- TAVASSOLI M., AOKI M. 1989. Localization of megakaryocytes in the bone marrow. *Blood Cells* **15**: 3-14.
- VAN-DER-LOO B., HONG Y., HANOCK V., MARTIN J. F., ERUSALINSKY J. D. 1993. Antimicrotubule agents induce polyploidization of human leukaemic cell lines with megakaryocytic features. *Eur. J. Clin. Invest.* **23**: 621-634.
- WALASZEK E. J., KOSCIS J. J., LEROY G. V., GEILING E. M. 1960. Studies on excretion of radioactive colchicine. *Arch. Int. Pharmacodyn. Ther.* **125**: 371-378.
- WŁODARSKI K. H., WŁODARSKI P. K. 1997. Does colchicine really induce bone formation in the rodent bone marrow? *Calcif. Tissue Int.* **61**: 165-167.
- WOLF J., KNIPLING L. 1995. Colchicine binding by the “isolated” β -monomer of tubulin. *J. Biol. Chem.* **270**:16809-16812.