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Melatonin in thrombocytogenesis.

INTRODUCTION

A normal adult subject produces some 180x10⁹ platelets (pl) every day (Harker and Fink, 1969). Pl formation and inflow follow a finely regulated physiological process. The pl production per megakaryocyte (mgc) increases with ploidy and ploidy results from the number of endomitotic nuclear replications.

The "stress pl production" (Tong et al. 1987) in response to acute thrombocytopenia by stress and the appearance of large intrasinusoidal cytoplasmic mgc process, termed "proplatelets" by Breckèr and De Bruyn (1976) and demonstrated by Radley and Scrufield (1980) may contribute to account for the production of pl, propl and macrothrombocytes.

The doubts on mgc ploidization and pl production and release, reflect on the inadequate therapeutic drug efficacy in patients with altered pl count. A helpful explanation can arise from the study of the influence that nervous and neuroendocrine system play on bone marrow (b.m.) function.

The Bone Marrow Vessels

The finely and delicately designed order of maturation and release of blood cells and plasma globulins into circulating blood is bound with the nerve fibers and blood vessels entering the b.m. In our opinion, it is at this level that melatonin (MLT) exerts its main physiological peripheral action.

The shafts of the long bones are penetrated by a nutrient artery and by one or two veins (Rheinelander, 1972). The nutrient artery branches off into ascending and descending branches, which coil around the central vein. Both nutrient artery and vein cross-connect with metaphyseal and epiphyseal artery and vein. At the endosteal surface a vessel network communicates with a periosteal venous network. According to Brookes (1964) the blood flows normally from the endosteum to periosteum.

A higher blood flow rate moves on in red than in yellow b.m. (Petrakis, 1954). MLT infusion into nembutalized rats produces a transient and limited reduction of b.m. pressure, as well as a moderate temperature increase (Di Bella: unpublished result). The intrameduallary pressure reflects the primary, respiratory and vasomotor arterial pressure fluctuations (Foà, 1936, Foà and Roizim, 1936; Michelsen 1967, 1968), according to animal speciès, to posture and to tonic and phasic muscle activity.

Cummings (1962) reported a b.m. blood flow of 0.51 ml/g/min. Since the cross-sectional area of the venous system is about 5 times that of the nutrient

meta-epi-periosteal arterias (Bargmann, 1930) the blood flow, through the venous vessel is approximately 5 times slower. Within the sinusoidal network is characterized by its large size and the extreme thinness of its walls that are lined by flat cells, with rare overlapping; without basement membranes or desmosomes (De Bruyn et al. 1966; Paese, 1956; Zamboni and Paese, 1961).

The Megakaryocyte In The Bone Marrow

Mgc lie adjacent to the sinus wall (Lichtman et al. 1978; Tavassoli, 1980; Tavassoli and Aoki, 1981), in close apposition to the entothelial cell, so that neither basal membrane nor adventitial cells are interposed (Weiss and Chen, 1975).

The marrow cells could likewise migrate into sinusoids through the cytoplasm of the endothelial cells (Campbell, 1972; De Bruyn et al. 1971; Foà and Roizin, 1936; Muto, 1976; Tavassoli, 1977; Throwbridge et al. 1982; Weiss, 1970). The cell traffic could be active or passive, i.e. secondary to the differential pressure through the sinusoidal wall.

Cell maturity (Giordano and Lichtman, 1973) and cell deformability (Le Blond et al. 1973; Lichtman 1970) interact differently with the sinus wall. In the normal rat femur b.m. mgc are randomly scattered all over the diaphysis with a minimum at the metaphyseal ends. The ratio between, the absolute number of rat b.m. mgc and the dry weight of the b.m. grows after chronic (3 months) i.p. MLT treatment (Di Bella: unpublished results). This increase may originate from a higher cellularity and a parallel reduction of the b.m. intercellular fluid, as well as from a rise of mgc density, without change of their distribution pattern.

The osmolarity of the supranatant intercellular fluid remains constant at ambient temperature up to 12 hrs; on the contrary the osmolarity of the whole rat b.m. increases by 70 mOsm/hr at ambient temperature (Di Bella et al. 1975a, 1975b). The deduction is put forward that some b.m. cells enjoy the peculiarity of taking up water from the surrounding medium, and/or secreting substances into the same fluid. The b.m. cell volume increases when the cells migrate into the sinusoid plasma; through this mechanism the cellular traffic becomes easy and occurs unidirectionally.

The osmotic gradient and the rhythmical pulse by the b.m. arteries are both momentous events for pl and/or mgc entrance into the blood current.

MLT plays a prevailing, manifold role in all these processes (Lemaitre et al. 1981), as is proven both by the histological evidence of b.m., and by the propitious clinical course of several blood diseases, after MLT treatment.

The Influence Of Melatonin On Megakaryocytes

Mgc show a peculiar behaviour in vitro. Nakeff (1977) demonstrated that

it was possible to study pl formation in plasma culture. The conditions for a successful culturing of colonies in a plasma culture system and the transfer to a slide for AChE staining, as a specific cytochemical marker have been established by several authors (Metcalf et al. 1975; Nakeff et al. 1975; Porter and Gengozian, 1972).

By adding APD or MLT or both to a suspension of b.m. cell the membranes of some mgc grow thinner, break and finally release a cluster of pl (Di Bella et al. 1979a, b; Harker and Fink, 1969; Rossi et al. 1979). The process resembles the formation of cytoplasmic fragments from the body of mgc, that flow through gaps of the sinusoidal membrane and turn into pl, although the whole mgc can enter the blood and stop within the pulmonary circulation (Kallinikos-Maniakis, 1969; Kaufman et al. 1965a, b; Pederson, 1978).

The Innervation Of Bone Marrow

A not yet settled problem concerns the physiological role that myelinated and non-myelinated fibers play in the b.m. Most fibers enter the bone hole through the nutrient canal; only a few fibers cross epi-, meta- and diphyseal foramina (Gros, 1846; Ottolenghi, 1902; Rossi, 1932; Variot and Remy, 1880). Most myelinated fibers are afferent: they convey pain sensation or are linked to yet unknown reflex arcs (Kuntz and Richins, 1945). Most sympathetic non-myelinated nerve fibers are probably tied to blood vessel diameter regulation; many free endings, such as brushes, rings, buttons or filaments are probably cholinergic (Miller and Kasahara, 1963).

Parenchymal nerve fibers are predominantly AChE positive, as well as some nerve fibers that are adjacent to large blood vessels (Calvo, 1968). Other nerve fibers in the adventitia of arteries and arterioles, or associated with venules, veins and sinusoids are on the contrary adrenergic (Miller and McCuskey, 1973).

The morphological differentiation of the marrow nerves is completed within the first two weeks of life in the rat, when the newborn rat begins to respond to erythropoietin (Garcia and Van Dyke, 1961), to starvation and hypoxia (Lucarelli et al. 1968). The coupling of these experimental reports has suggested the speculation that hematopoiesis enjoys a neuro-humoral control, and that the cells released from the b.m. are controlled by sympathetic fibers (Di Pace et al. 1975).

ß-adrenergic agonists increase erythropoiesis even in polycytemic mice (Fink and Fisher, 1977 a, b), whereas the ß-adrenergic antagonist propanolol blocks the reaction (Brown and Adamson, 1977; Brown et al. 1976; Gangar et al 1975). ß-adrenergic receptors have been found even on pluripotent stem cells (Byron, 1972). The stimulation of the sympathetic lumbar chain promotes the release both of retyculocytes and neutrophils (Di Pace and Webber,

1975). Manometric methods have proved the presence of AChE in erythrocytes, pl and plasma in different animal species (Augustinsson et al. 1952; Zajicek and Datta, 1953). Koelle (1951) has found sites of AChE activity at the surface of pl ghosts and erythrocytes. Zajicek (1954) showed that rabbit and rat pl and mgc contained relavitely high amounts of AChE. AChE was found also in embryonic (Minganti and Falugi, 1980) and cancerous cells (Falugi et al. 1983), so that an involvement of the enzyme has been postulated in the regulation of plasma membrane functions (Finin et al. 1979; Gandin et al. 1979). AChE is apparently a marker for early cells of the megakaryocytes series.

By adding MLT to a suspension of b.m. cells, cluster of pl can appear on mgc membrane. Orally or i.m. MLT (about 0.2 mg/Kg b.w./day) may heal some thrombocytopenic patients, and cure hemopathic and cancerous patients.

The electrical stimulation of the habenular ganglia brings about a transitory but significant rise of the blood pl count (Di Bella et al. 1979c).

When MLT is added to a fresh b.m. cell suspension together with inhibitors of NAT or HIOMT, many fluorescent pl appear packed against the membrane of mgc, without protruding or leaving it. The size of the pl that form in these conditions is bigger following NAT- than HIOMT-inhibitors.

The preceding findings can be accounted for when one admits that:

1) a NAT, or NAT-isozyme that is responsive to NAT inhibitor chemicals, exists in the mgc membrane;
2) a HIOMT, or HIOMT-isozyme that is responsive to HIOMT-inhibitor chemicals, exists in the mgc membrane;
3) the topical inhibition of either NAT or HIOMT enzymes or isozymes is associated with the formation of pl in the mgc membrane;
4) when NAT or HIOMT-inhibitors are lacking, MLT crosses the mgc membrane (Lemaitre et al. 1981), and promotes pl formation at the level of the demarcation membrane system. The mgc membrane grows here more and more thin and then disappears where pl take shape and crowd.
5) The results of MLT action are equivalent to those of NAT or HIOMT inhibitors. Indeed in the reactions:
   (1) Serotonin+Methyldonors Methoxyserotonin
   (2) Methoxyserotonin+Acetyldonors Melatonin
   the velocity ot the forward reactions is decreased and the velocity of the reverse reaction is raised by MLT, so that the availability of the acetyldonors and methyldonors is raised by MLT.

MLT that does not react either in the cell membrane or in the demarcation membranes of mgc reaches the nucleus. At this level MLT could exert a fi-

cytochalasin-like action so as to inhibit the process of endoreduplication and increase the modal ploidy.

CONCLUSIONS

1) The role of MLT in thrombocytogenesis is essential, although not yet clearly defined.
2) MLT therapeutic failure in some thrombocytopenic patients originates from a deficiency of some other factors which mediate the activity of MLT.
3) The pl formation can therefore be associated with the synthesis of alkylacetylglycerol, of alkylacetylglycerophosphocholine and of pl activating factor (PAF). The cellular ubiquity and the extent of the reactions catalyzed by PAF, as well as the high meaning of the reactions that pl displays with the vessel wall, throw sufficient light upon the universal and essential role of melatonin.

REFERENCES

1) Augustinsson, K.B., Datta, N., Grahn, N. and Zajicek, J. (1952), Acetylcholinesterase in thrombocytes. *Nature*, **169**, 800.
2) Bargmann, W. (1930), Uber den Feinbau der Knochenmarks-Kapillaren. *Z. Zellforsch-mikrosk. Anat.*, **11**, 1.
3) Battistini, N., Rossi, M.T., Scalega G., Di Bella L., Ciccolo F. (1975), Numero di elementi e di megacariociti del midollo osseo di ratto. *Boll. Soc.it. Biol. Sper.*, **51**, 18bis, 21.
4) Brecker, R.P. and De Bruyn, P.P.H. (1976): Tjhe transmural passage of blood cell into myeloid sinusoids and the entry of platelets into the sinusoidal circulation: A scanning electron microscopic investigation. *Am. J. Anat.*, **145**, 183.
5) Brookes, M. (1964), in: Clark, J.PM.P. (ed.) Modern trends on orthopaedics, Vol. 4, *Butterworths, London*, **p. 91**.
6) Brown, J.E. and Adamson, J.W. (1977), Modulation of in vitro erytropoiesis. The influence of ß-andrenergic agonists on erythroid colony formation. *J. Clin. Invest.*, **60**, 70.
7) Brown, E.M., Fedak, S.A., Woodward, C.J. (1976), ß-adrenergic receptor interactions. Direct comparison of receptor interactions and biological activity. *J. Biol. Chem.*, **251**, 1239.
8) Byron, J.W. (1972), Evidence for a ß-adrenergic receptor initiating DNA synthesis in haemopoietic stem cells. *Exp. Cell. Res.*, **71**, 228.
9) Calvo, W. (1968), The innervation of the bone marrow in laboratory animals. *Am. J. Anat.*, **123**, 315.
10) Campbell, F.R. (1972), Ultrastructural studies of the transmural migration of blood cells in the bone marrow of rats, mice and guinea pigs. *Am. J. Anat.*, **135**, 521.
11) Cummings, J.D. (1962), A study of the blood flow through the bone marrow by a method of venous effluent collection. *J. Physiol., London*, **162**, 30.
12) De Bruyn, P.P.H., Michelsen, S. and Thomas, T.B. (1971), Migration of blood cells of the bone marrow through the sinusoidal wall. *J. Morphol.*, **133**, 417.
13) De Bruyn, P.P.H., Thomas, T.B. and Michelsen, S. (1966), Fine structure of the vascular components of the guinea pig bone marrow. *Anat. Rec.*, **154**, 499.
14) Di Bella L., Battistini, N., Coronati, R., Rossi, M.T. e Scalera, G. (1975a), Sulla pressione osmotica del midollo osseo. *Boll. Soc. it. Biol. Sper.*, **51**, 18bis, 25.

15) Di Bella, L., Gualano, L., Rossi, M.T., Scalera, G. (1979a), Azione della Melatonina (MLT) sulla piastrinogenesi in vitro. *Boll. Soc. it. biol. Sper.*, **55**, 323.
16) Di Bella, L., Gualano, L., Rossi, M.T., Scalera, G. (1979b), Effetti dell'azione simultanea della Melatonina (MLT) e dell'ADP sui megacariociti in vitro. Boll. *Soc. it. Biol. Sper.*, **55**, 389.
17) Di Bella, L., Rossi, M.T. and Scalera, G. (1979c), Perspectives in Pineal Functions. In: J. Ariëns Kappers & P. Pévet (Eds). The Pineal Gland of Vertebrates including Man (Progr. Brain Res., vol. 52), *Elsevier/North-Holland Biochemical Press, Amsterdam*, 475.
18) Di Bella, L., Scalera, G., Battistini, N., Coronati, R. e Rossi, M.T. (1975b), Influenza della componente cellulare di midollo sopra la pressione osmotica del liquido intercellulare. *Boll. Soc. it. Biol. Sper.*, **51**, 18bis, 23.
19) Di Pace, D.M. and Webber, R.H. (1975), Electrostimulation and morphologic study of the nerves of the bone marrow of the albino rat. *ACTH Anat.*, **93**, 1.
20) Falugi, G., Balza, F., Zardi, L. (1983), Localization of acetylcholinesterase in normal human fibrosarcoma cell line. *Bas. Appl. Histochem.*, **27**, 205.
21) Finin, V.S., Volotovskii, I.D. and Konev, S.V. (1979), Role of acetylcholinesterase in the transmembrane transfer of anions in erythrocytes. *Biofizika*, **24**, 96.
22) Fink, G.D. and Fisher, J.W. (1977a), Stimulation of erythropoiesis by ß-adrenergic agonists. I. Characterization of activity in polycythemic mice. *J. Pharmacol. Exp. Ther.*, **202**, 192.
23) Fink, G.D. and Fisher, J.W. (1977b), Stimulation of erythropoiesis by ß-adrenergic agonists. I. Mechanisms of action. *J. Pharmacol. Exp. Ther.*, **202**, 199.
24) Foà, P. (1935), Sull'innervazione funzionale del midollo delle ossa. *Arch. Sc. Biol.*, **21**, 113.
25) Foà, P. and Roizin, L. (1936), Sull'innervazione funzionale del midollo delle ossa. Nota II. *Arch. Sc. Biol.*, **22**, 595.
26) Gadin, D.V., An, T. Garnett, M.E. (1979), Acetylcholinesterase: a probe for the study of anti-arhythmic drug membrane interactions. *Biochim. Biophys. Acta.*, **512**, 388.
27) Ganger, D., Kaiser, E., Quiring, K., Palm, D. (1975), The ß-adrenergic receptor-adenylciclase system of rat: retyculocyte effect of adrenergic stimulants and inhibitors. *Naunyn-Schmiedeberg Arch. Pharmacol.*, **289**, 379.
28) Garcia, F.C. and Van Dyke, D.C. (1961), Responses of rats of various ages to erythropoietin. *Prot. Soc. Exp. Biol. Med.*, **106**, 585.
29) Giordano, G.F. and Lichtman, M.A. (1973), Marrow cell egress: the central interaction of barrier pore size and cell maturation. *J. Clin. Invest.*, **52**, 1154.
30) Gros, M. (1846), Note sur les nerfs des os. *C.R. Acad. Sci.*, **23**, 1100.
31) Gualano, L., Di Bella, L., Rossi, M.T., Scalera, G. (1979), Contributo alle tecniche di studio in vitro dei megacariociti. *Boll. Soc. it. Biol. Sper.*, **55**, 318.
32) Harker, L.A. and Fink, C.A. (1969), Thrombokinetics in man. *J. Clin. Invest.*, **48**, 963.
33) Hashimoto, M. (1935), Uber das gröbere Blutgefässystem des Kaninchenknochenmarks. *Trans. Soc. path. jap.*, **25**, 371.
34) Hashimoto, M. (1936), Uber das kapillare Blutgefässystem des Kaninchenknochenmarks. *Trans. Soc. path. jap.*, **26**, 300.
35) Irino, S. (1975), Stereo-microvascular architecture and transmural migration of blood cells in the bone marrow as revealed by scanning electron microscopy. *Acta Haematol. jpn.*, **38**, 647.
36) Kallinikos-Maniakis, A. (1969), Megakaryocytes and platelets in central venous and arterial blood. *Acta Haematol.*, **42**, 330.
37) Kaufman, R.M., Airo, R., Pollack, S. and Crosby, W.H. (1965a), Circulating megakaryiocytes and platelet release in the lung. *Blood*, **26**, 720.
38) Kaufman, R.M., Airo, R, Pollack, S., Crosby, W.H. and Doberneck, R. (1965b), Origin of pulmonary megakaryocytes. *Blood*, **25**, 765.

39) Koelle, G.B. (1951), The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. *J. Pharmacol.*, **103**, 153.
40) Kuntz, A. and Richins, C.A. (1945), Innervation of the bone marrow. *J. Comp. Neurol.*, **83**, 213.
41) Le Blond, P.F., La Celle, P.L. and Weed, R.I. (1973), Cellular deformability: a possible determinant of the release of maturating erythrocytes from the bone marrow. *Blood*, **27**, 40.
42) Lemaitre, B.J., Leunay, J.M., Dreux, C., Hartman, L. and Da Prada, M. (1981), Melatonin content, uptake and synthesis by blood platelets. EPSG 2nd Colloq. Giessen, 42.
43) Lichtman, M.A. (1970), Cellular deformability during the maturation of myeloblast: possible role in marrow egress. *N. Engl. J. Med.*, **283**, 943.
44) Lichtman, M.A., Chamberlain, J.K., Simon W. and Santillo P.A. (1978), Parasinusoidal location of megakaryocytes in the marrow: a determinant of platelet release. *Am. J. Hematol.*, **4**, 303.
45) Lucarelli, F., Porcellini, A., Carnevali, C. (1968), Fetal and neonatal erythropoiesis. *Ann. N.Y. Acad. Sci.*, **149**, 544.
46) Metcalf, D., MacDonald, H.R., Odartchenko, N., Sordat, B. (1975), Growth of mouse megakaryocyte colonies in vitro. *Proc. Natl. Acad. Sci.*, USA, **72**, 1744.
47) Michelsen, K. (1967), Pressure relationship in the bone marrow vascular bed. *Acta Physiol. Scand.*, **71**, 16.
48) Michelsen, K. (1968), Hemodinamics of the bone marrow circulation. *Acta Physiol. Scand.*, **73**, 264.
49) Miller, M.R. and Kasanara, M. (1963), Observation on the innervation of human long bones. *Anat. Rec.*, **145**, 13.
50) Miller, M.L. and McCuskey, R.S. (1973), Innervation of bone marrow in the rabbit. *Scand. J. Haemat.*, **10**, 17.
51) Minganti, A. and Falugi, C. (1980), An epithelial localization of acetylcholinesterase in the ascidian Ciona intestinalis embryos and larvae. *Acta Embryol. Morphol. Exp.*, **1**, 143.
52) Muto, M. (1976), A scanning and transmission electron microscopic study on rat bone marrow sinuses and transmural migration of blood cells. *Arch. Histol. jpn.*, **39**, 51.
53) Nakef, R. (1977), Colony-forming unit megakaryocyte (CFU-M). In:S.J. Baum & D.G. Ledney (Eds), *Experimental hematology today, Springer, Berlin*, 111.
54) Nakeff, R., Dicke, K.A. and Van Noord, M.J. (1975), Megakaryocytes in agar cultures of mouse bone marrow. *Series haematol.*, **8**, 4.
55) Ottolenghi, D. (1902), Sur les nerfs de la moelle des os. *Arch. it. Biol.*, **37**, 73.
56) Paese, D.C. (1956), Electron microscopic study of the rat bone marrow. *Blood*, **11**, 501.
57) Pederson, N.T. (1978), Occurrence of megakaryocytes in various vessels and their retention in the pulmonary capillaries in man. *Scand. J. Haematol.*, **21**, 369.
58) Petrakis, N.L. (1954), Bone marrow pressure in leukemic and non-leukemic patients. *J. Clin. Invest.*, **33**, 27.
59) Porter, R.P. and Gengozian, N. (1972), In vitro proliferation of heme precursor cells from marrow and blood of naturally chimeric marmosets. *J. Cell. Physiol.*, **79**, 27.
60) Radley, J.M. and Scurfield, G. (1980), The mechanism of platelet release. *Blood*, **56**, 996.
61) Rhinelander, F.M. (1972), Circulation in bone. In: The biochemistry and physiology of bone, Vol. II, *Academic Press, N.Y.*, 1.
62) Rossi, F. (1932), L'innervazione del midollo osseo. *Arch. It. Anat. Embryol.*, **29**, 539.
63) Rossi, M.T., Gualano, L., Di Bella, L., Scalera, G. (1979), Azione dell'ADP sulla piastrinogenesi in vitro. *Boll. Soc. it. Biol. Sper.*, **55**, 327.
64) Rossi, M.T., Scalera, G., Di Bella, L., Battistini, N., Tarozzi, G. (1975), Volume ed impacchettamento cellulare del midollo osseo. *Boll. Soc. it. Biol. Sper.*, **51**, 18bis, 22.

65) Tavassoli, M. (1977), Adaptation of marrow sinus wall to fluctuation in the rate of cell delivery: studies in rabbit after blood letting. *Brit. J. Haematol.*, **35**, 25.
66) Tavassoli, M. (1980), Megakaryocyte-platelet axis and the process of platelet formation and release. *Blood*, **55**, 537.
67) Tavassoli, M. and Aoki, M. (1981), Migration of entire megakaryocytes through the marrow-blood barrier. *Brit. J. Haematol.*, **48**, 25.
68) Tong, M., Seth, P. and Penington, D.G. (1987), Proplatelets and stress platelets. *Blood*, **69**, 522.
69) Throwbridge, E.A., Martin, J.F. and Slater, D.N. (1982), Evidence for a theory of physical fragmentation of megakaryocytes implying that all platelets are produced in the pulmonary circulation. *Thromb. Res.*, **28**, 461.
70) Variot, G. and Remy, C. (1880), Sur les nerfs de la moelle des os. *J. Anat. Physiol.*, **6**, 273.
71) Weiss, L. (1970), Transmural cellular passage in vascular sinuses of rat bone marrow. *Blood*, **36**, 189.
72) Weiss, L. and Chen, L. (1975), The organization of the hematopoietic cords and vascular sinuses in the bone marrow. *Blood Cells*, **1**, 617.
73) Zajicek, J. (1954), Studies on the histogenesis of blood platelets. *Acta Haemat.*, **12**, 238.
74) Zajicek, J. and Datta, N. (1953), Investigation on the acetylcholinesterase activity of erythrocytes, platelets and plasma in different animal species. *Acta Haemat.*, **9**, 115.
75) Zamboni, L. and Pease, P.C. (1961), The vascular bed of the red bone marrow. *J. Ultrastruct. Res.*, **5**, 65.