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Erythrophagocytosis by cultured skin fibroblasts from patients with hereditary metabolic disorders

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Abstract. Phagocytosis of native allogenic red blood cells was observed in cultures of skin fibroblasts obtained from patients with neuronal ceroid-lipofuscinosis, Niemann-Pick disease type C and morbus Fabry. Occasional phagocytizing cells were observed in 9 other syndromes. Cells from three normal donors did not phagocytize.
Key words. Erythrophagocytosis; metabolic disorders; cultured skin fibroblasts.

Phagocytosis of autologous red blood cells (RBC) and formation of specific lipopigments was previously described in appendical macrophages from patients with neuronal ceroid-lipofuscinosis (NCL)[1]. Subsequently, erythrophagocytosis of allogenic RBC without formation of specific curvilinear bodies was observed in cell lines of skin fibroblasts isolated from skin biopsies obtained from NCL patients[2]. It has been suggested that the capacity for erythrophagocytosis is closely related to alteration of the fibroblast.

The aim of this communication is to present further studies on erythrophagocytosis by fibroblast cell lines. This phenomenon has not been widely studied so far. Here, we evaluate the RBC phagocytic capacity in a series of 16 skin fibroblast cell lines representing other metabolic and degenerative disorders and controls.

Material and methods

Skin biopsies were obtained from 16 patients with a specific diagnosis. Two patients had neuronal ceroid lipofuscinosis (NCL) (late infantile and juvenile type), diagnosed by excessive generalized deposition of the disease-specific lipopigment[3]. Three patients with Niemann-Pick disease type C were diagnosed by histochemistry and electron microscopy[4], and by the substantially decreased capacity for cholesterol esterification of cultured fibroblasts (Dr. M. T. Vanier, Lyon). Further examples examined were: Fabry disease (two cases), Krabbe disease (one case), GM1 and GM2 gangliosidosis (one case each), Zellweger syndrome (one case), mevalonic aciduria, Edwards and Coffin-Lowry syndromes, and two nonspecified neurodegenerative disorders. Fibroblasts from three healthy young adults were used as controls.

Cell lines established from skin biopsies and stored in liquid nitrogen in the 4th–10th passage were thawed and multiplied at 36–37 °C in Eagle's MEM containing 10% fetal calf serum, 250 μg glutamine/ml and 40 μg gentamycine/ml of culture medium. Confluent cultures were trypsinized with 0.05% trypsin (DIFCO Trypsin 1:250, DIFCO, USA) and 12.5×10^3 cells in 2.5 ml medium were placed on glass tissue culture chamber slides[5] and incubated for 6 h in a humidified 5% CO_2 atmosphere. The medium was then replaced by the same amount of fresh medium containing a suspension of allogenic RBCs. This was prepared by putting one drop of fresh native (non-heparinized) blood obtained from one constant donor (blood group B, Rh+) into the culture medium. The volume of medium was then adjusted to a final cell concentration of 1×10^5 RBCs/ml. After 2, 4 and 7 days of incubation, the cylinders forming the chambers were removed and the slides were thoroughly washed in PBS, fixed with acetone-methanol and stained with Giemsa. The number of phagocytizing cells per 1000 cultured cells and the number of phagocytized RBCs were counted by optical microscopy using at least 10 randomly-chosen fields at 600× magnification for the fibroblasts and 1000× magnification for detailed evaluation of phagocytized RBCs. Cells cultured under the same conditions were used for enzyme-histochemical and immunocytochemical investigation. Acid phosphatase was demonstrated by simultaneous azocoupling (naphthol-ASBI derivatives, hexazonium p-rosaniline) and nonspecific esterase (α-naphthyl acetate, hexazonium p-rosaniline). Macrophage markers (lysozyme, α-1-antitrypsin – AAT, α-1-antichymotrypsin – ACHT and myeloid – histiocyte antigen – MAC 387) were examined by an indirect immunoperoxidase technique. The cultures were fixed with acetone, washed 3 times with PBS and incubated with primary antibodies. As the second antibody horseradish peroxidase (HRP) conjugated F(ab')2, fragments of swine anti-rabbit (for lysozyme, AAT and ACHT) and swine anti-mouse (for MAC 387) were used. The immunoreactivity product was visualized with diaminobenzidine.

Percentage of phagocytizing cells and total number of internalized red blood cells (in parentheses) in cultured skin fibroblasts of 16 patients with hereditary syndromes and 3 normal donors

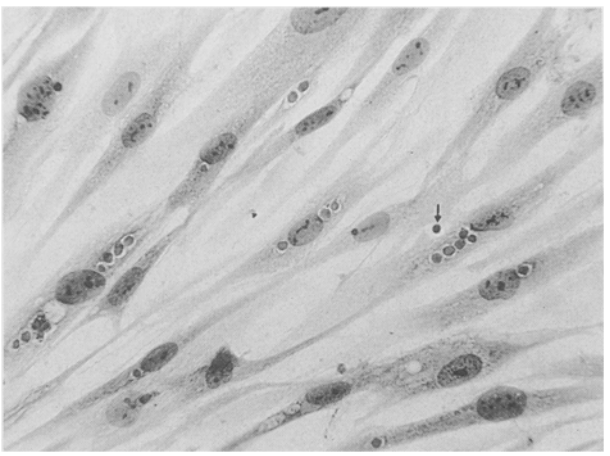
Syndrome	Patient	Age	4 days' incubation	7 days' incubation
NCL	B. P.	10 yrs	1% (20) n = 1008	20% (354) n = 999
	M. C.	5 yrs	17% (529) n = 1002	6.6% (145) n = 1004
NPC	L. M.	4 yrs	17% (146) n = 1000	0.3% (3) n = 999
	S. I.	10 yrs	17% (432) n = 1004	8% (239) n = 1010
	P. K.	3 yrs	3% (74) n = 966	17% (541) n = 1044
Fabry	K. V.	17 yrs	15% (219) n = 986	17% (243) n = 971
	P. C.	16 yrs	8% (132) n = 1012	5% (294) n = 1037
Krabbe	D. P.	1 day	1.6% (21) n = 1053	3.6% (77) n = 1037
Zellweger	R. K.	4 yrs	1% (19) n = 1119	2% (41) n = 1131
C-Ls	M. P.	6 yrs	0.4% (5) n = 1033	0.4% (3) n = 998
MVa-uri	H. S.	3 yrs	0.6% (6) n = 1020	0.1% (30) n = 1420
GM 1	I. C.	unknown	0.4% (4) n = 1005	0.09% (1) n = 1002
GM 2	M. H.	3 yrs	0% (0) n = 1008	0% (0) n = 997
ES	I. H.	1 day	0.3% (4) n = 996	- - -
NSNDs	M. G.	8 months	0% (0) n = 995	0.8% (16) n = 1005
	M. H.	2 yrs	0.8% (16) n = 1011	- - -
Control	M. S.	23 yrs	0% (0) n = 1025	0% (0) n = 1001
	S. B.	21 yrs	0% (0) n = 999	0% (0) n = 1028
	R. V.	43 yrs	0% (0) n = 1022	0% (0) n = 1005

Abbreviations: NCL neuronal ceroid lipofuscinosis, NPC Niemann-Pick type C disease, C-Ls Coffin-Lowry's syndrome, MVa-u mevalonic aciduria, ES Edwards syndrome, NSNDS nonspecified neurodegenerative disorder. - not done, n = number of cells examined.

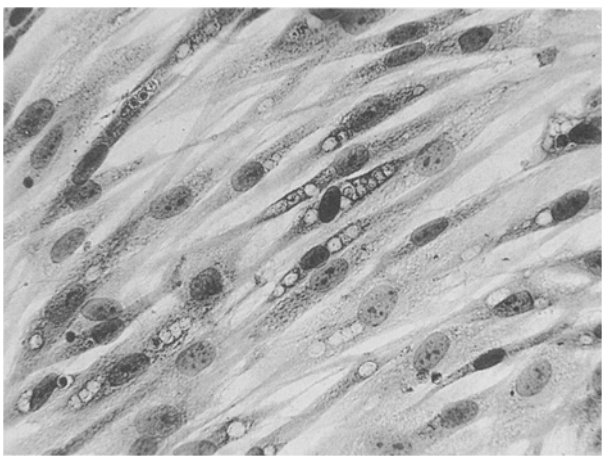
Results

As shown in the table, RBC phagocytosis was seen in cultured cells from patients with NCL, Niemann-Pick type C and Fabry's disease, without substantial difference among them. Cells from other disorders tested displayed varying degrees of RBC ingestion, but generally the phenomenon was much less extensive. No RBC phagocytosis was seen in cultured cells obtained from normal donors. The RBC phagocytosis began after two days' incubation and was maximal from the fourth to seventh day of culture, when the experiment was terminated due to confluence of the cultures (table). RBC phagocytizing capacity was never uniform. RBC-loaded elements were located in nests dispersed among inactive cells of the same morphology. Various states of RBC digestion, ranging from apparently intact interiorized RBCs, through amorphous remnants to empty phago-lysosomal vacuoles, could be seen in both native and stained preparations (fig. A, B). The cytoplasm of the phagocytizing cells from the most "active" cases was often entirely filled with ingested RBCs in different stages of degradation. The phagocytizing cells seen occasionally in the other less active disorders studied rarely contained more than two RBCs.

Cell cultures displaying RBC phagocytosis were tested for the presence of histiocytic cells. Enzyme histochemistry showed medium strength acid phosphatase activity with some minor variation and slight activity of nonspecific esterase. Immunohistochemical markers for histiocytes (lysozyme, AAT, ACHT, MAC387) were negative. This enzyme-histochemical pattern and immunophenotype was identical in all cell lines investigated, including control cultures.



A Niemann-Pick type C. Ingested RBCs in fibroblasts after 4 days of culture. Arrow: initial stage of RBC internalization. Giemsa, 200 ×.



B The same as in *A*. Advanced stage of RBC digestion. Giemsa, 200 ×.

Discussion

The results obtained lead to the conclusion that RBC phagocytosis by cultured skin fibroblasts is a nonspecific disease-induced phenomenon. The diseases inducing the most intense RBC phagocytosis belong to the broad family of lysosomal storage disorders, even if the nature of the metabolic defect in two of them, NCL and Niemann-Pick type C, has not been explained satisfactorily so far[6,7]. Significantly less RBC phagocytosis induction was observed in other disorders examined (see table). Results from NCL mutant cells corroborated our previous findings in this disease[2], suggesting the effect is widespread.

Formal aspects of the RBC phagocytosis process, worth mentioning, are as follows. Internalization of RBCs, seen by microcinematography only after 18 hours' contact[2] was clearly apparent after 4 days of observation (hence data for earlier intervals are not included in this study). RBC digestion was completed within the experimental period, leaving only empty digestion vacuoles without any detectable residual material.

The expression of RBC phagocytosis by only a fraction of the cultured cells (see table) points to a functional heterogeneity, previously described for instance for cultured fibroblast glycosaminoglycan metabolism[8] or collagen synthesis[9]. It is highly improbable that admixture of phagocytic histiocytes is a cause of cultured cells' heterogenous RBC phagocytic potential. The cultured cells (derived from controls, or from patients with NCL or NPC) did not display higher activities of nonspecific esterase and acid phosphatase, nor any of the immunocytochemical macrophage markers (lysozyme, AAT, ACHT, MAC387). The repeated trypsinization involved in passaging of our cell lines could also contribute to the elimination of macrophages from the cultures.

We suggest, therefore, that the nests of phagocytizing cells represent a clonal expansion of functionally modified true dermal fibroblasts. The nature of the mechanism responsible for the functional modulation is unclear, but we assume that it may be common for all the disorders studied. It might reflect altered cell membrane composition related to the metabolic error. This in turn might cause increased RBC adhesion, thereby triggering phagocytosis. Alteration of cell membranes has been described in NCL[10,11]. The age of the donors does not seem to play a role. The donors of normal skin samples were young adults, while the majority of our patients were children. However, normal human embryonic lung fibroblasts did not reveal phagocytosis in our previous study[2].

The origin of RBCs used for the experiments did not seem to influence the process of phagocytosis. The phagocytic activity of fibroblasts obtained from the patients with NCL to the erythrocytes of blood group O, Rh−, which were used on our original study[2], was comparable with the phagocytosis of erythrocytes of blood group B, Rh+, described in this paper.

To our knowledge no other report has dealt with RBC phagocytosis in cultures of modified fibroblasts isolated from other hereditary or acquired metabolic disorders. The only reports on phagocytic properties of the fibroblasts deal with their normal capacity to ingest collagen both in vivo and in vitro[12] which seems to reflect local collagen turnover. On the other hand, the capacity for erythrophagocytosis in vivo has already been described in several types of epithelial cells under specific pathological conditions[13,14].

The substrate specificity of induced phagocytosis and its relation to basic fibroblast functions, e.g. collagen and glycosaminoglycan synthesis and turnover, remain to be established. Last but not least it will be important to know if the phenomenon occurs in vivo.

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