Evolutionary intraembryonic origin of vertebrate hematopoietic stem cells in the elasmobranch spleen

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Abstract

The electric ray (*Torpedo marmorata* Risso) provides an animal model for the detection of early intraembryonic hemopoietic stem cells (HSCs) in sea vertebrates. The spleen of this bone-marrowless vertebrate appears to be the major site of HSCs differentiation during development and in adulthood. Splenic development in this species was investigated and hemopoietic stem cells were detected in this organ by immunocytochemistry utilizing CD34 and CD38 antibodies. At stage I (2-cm-long embryos with external gills), the spleen contains only mesenchymal cells. At stage II (3-4-cm-long embryos with a discoidal shape and internal gills), an initial red pulp was observed in the spleen, without immunostained cells. At stage III (10-11-cm-long embryos), the spleen contained well-developed white pulp, red pulp and ellipsoids. Image analysis at stage III showed four cell populations, *i.e.*, CD34+/CD38-, CD34+/CD38+, CD34-/CD38+, and CD34-/CD38- cells. The present findings, obtained from an elasmobranch, indicate that the CD34 and CD38 phenotypes are conserved through vertebrate evolution.

Introduction

The development of hemopoietic stem cells (HSCs) begins during early embryonic life and the embryos offer an insight into the complex characteristics and regulation of these cells.[1] There is conservation of the overall hematopoietic process between vertebrates, although some differences about the site of hemopoiesis do exist.[2] In bone marrowless vertebrates the spleen is a primary hemopoietic organ[3] and the main secondary lymphoid organ in mammals, in which it can also support bone marrow hemopoiesis in various conditions.

In the evolutionary tree, the spleen first appears as a separate organ in cartilaginous fish, in which the splenic tissue is an outgrowth of the mesenchyme traversed by sinusoidal capillaries.[4] In the adult electric ray, a viviparous elasmobranch, the spleen is located in the gut mesentery, and is round-shaped and slightly lobed.[5] It consists in red pulp, where the thrombopoiesis and erythopoiesis occur, and in white pulp, where lymphocytes and plasma cell maturation takes place.[6]

In some species, the limits between the white pulp and the red pulp are hardly identifiable, but, after antigenic stimulation, distinguishable lymphoid aggregates appear. These data indirectly suggest that the spleen is the site for antibody synthesis in elasmobranchs.[6,7]

In adult specimens of *Torpedo marmorata* Risso, the spleen is slightly triangular, flattened and located between the stomach and the duodenum, and also in contact with the pancreas. In this animal weighing 800 g, the spleen is 2.5 cm long, 1.5 cm wide and 0.5 cm thick.[8] The elasmobranchean spleen accounts for 0.21-0.36% of the body weight (even reaching 2.5% in some large-sized sharks), a relative size almost comparable to that of man, in whom the spleen is 0.27% of the body weight.[9,10] The elasmobranch's spleen is encased in a thin capsule which contains broad, strong collagenous and fine elastic fibers, as well as fibrous septa which extend from the capsule to the red pulp and penetrate the white pulp.[11] We have previously described hemopoietic functions and the cell types of the torpedo spleen,[9] whereas only limited data (summarized in Table 1) are available on the embryonic development of the elasmobranch's spleen. Splenectomy in torpedoes was found to result in an immediate dramatic decrease of hematological values, followed by normalization of the values for the red series and by the appearance of immature cells of all lineages in the blood circulation.[10] The latter data suggested the occurrence of circulating stem cells.

Despite the interest in this animal model, no studies of the HSCs have been performed in elasmobranchs thus far. We have indirectly demonstrated the occurrence of circulating HSCs in the marbled electric ray by revealing hemopoietic regeneration in the spleen and lymphomyeloid tissues after sublethal X-irradiation followed by autohemotransplantation.[8] Although several reports have described the histogenesis of hemopoietic organs in a variety of vertebrate species, the origin of HSCs in vertebrates is still a matter of debate. It is well known that vertebrate hematopoiesis involves two waves: the primitive transitory wave producing red blood cells to oxygenate the developing embryo and the definitive wave which occurs at different time points in different species, that produces erythroid-myeloid progenitors.[12,13] Definitive hematopoiesis later involves HSCs, which are multipotent and give rise to all blood lineages of the adult organism.[14]

It is established that mammalian HSCs possess the CD34+/CD38- phenotype, and these cells are believed to represent the earliest active multipotential stem cells. The murine adult bone marrow CD34+ cells were capable of long-term hematopoietic reconstitution and allow survival of lethally irradiated mice.[15-17] Contrarily, CD34- HSCs exist as well and are recognized as quiescent stem cells. To date the cell surface adhesion molecule CD34 is the most used marker for HSCs. The CD34 antigen, expressed on 1-5% of mononuclear bone marrow cells, has been shown to possess colony-forming potential in short-term assays and to maintain long-term colony forming potential in *in vitro* cultures.[18]

Early ontogenesis of HSCs in the spleen of elasmobranchs is unknown, however CD34+ cells were detected in other aquatic animals. Cima *et al.*[19] reported the morpho-functional characterisation of hemoblasts in bivalve mollusc *Tapes philippinarum*.

These cells have the typical characteristics of undifferentiated elements, with a high nucleus:cytoplasm ratio and no enzyme activity. Moreover, the hemoblasts were positive for the anti-mouse CD34 monoclonal antibody (known to identify haemopoietic cells in mammals). This supports the hypothesis that hemoblasts represent stem cells, freely circulating in the blood in *T. philippinarum.*[19] CD34 antigen was detected in hematopoietic cells of the bone marrow and kidney of *Lithobates catesbeianus*, while it was absent in endothelial cells, thus suggesting that this molecule is phylogenetically restricted to the hematopoietic lineage. The above analysis was conducted by using monoclonal mouse anti-CD34 (Dako, Carpinteria, CA, USA; M7165).[20] CD34+ cells were discovered also in an angioleiomyoma diagnosed in a *Conger conger*. The immunohistochemical analysis of this tumor showed cell positivity to rabbit monoclonal anti-CD34 (clone QBEnd10). The CD34 exhibited intense immunoreactivity on the plasma membrane.[21]

On this basis, the present study was aimed at demonstrating the onset of CD34+ HSCs in the embryonic spleen of torpedoes and establishing in which stage of embryonic development CD34-immunoreactive cells can be detected in this organ.

Materials and Methods

The study was based on three embryonic stages of torpedoes (described in Table 2). The embryos were removed by cesarian section from a total of six pregnant torpedoes (3 *Torpedo marmorata* Risso and 3 *Torpedo ocellata* Rafinesque). Three embryos per stage were used. In addition, two adult torpedoes (one male and one non-pregnant female, weighing 800 g) and the bone marrow of two newborn rats were used as controls. All the torpedoes, caught in the Gulf of Naples during June to October, 2000, were kindly provided by the Zoological Station A. Dohrn (Naples, Italy). The characteristics of the animals used are summarized in Table 3.

All the specimens were narcotized with MS222, 62 mg/L (Sigma, St. Louis, MO, USA). The embryos were fixed in toto (see below). The spleens were excised from the three embryos at stage III and from the adult animals. The experiments were performed under institutional approval and all efforts were made to avoid animal suffering.

Histology

Embryos at stages I and II (n=3 per stage), spleens at stage III (n=3) and spleens from adult animals (n=2) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 30% sucrose, and paraffin-embedded. A small portion of the spleen at stage III and adult specimens was used to perform touch preparations

Table 1. Spleen development in elasmobranchs.

Author	Year	Elasmobranch species	Main characterstics of spleen development
Ruffini	1904	*Pristinus melanostomus, Scyllium canicula, Torpedo ocellata and Raja clavata*	First bud of the spleen appears on the dorsal mesentery, dorsally to the bud of the pancreas.
Scammon	1911	*Squalus acanthias*	In 24.7 mm embryos the spleen mesenchymal bud is 0.5 mm in length. In 34 mm embryos it attains 1.5 mm in length.
Ranzi	1932	*Torpedo marmorata*	The embryo of elongated shape, not yet flattened, weights 14 g.
Zapata	1996a	Dogfish	Lymphoid colonization takes place in 50-100 mm embryos with internal yolk sac.
Zapata	1996b	Squaliformes	Lymphoid colonization takes place in the spleen when the external gills are included.

Table 2. Characteristics of the three embryo stages.

Embryo stage	Shape	Total Size	Body size	Dorsal color	External organs	Gills	Yolk sac
I	Elongated	2 cm	2 cm	Milky-white	Well developed	External	External
II	Discoidal flattened	3.5-4.5 cm	1.5-2 cm	Light brown	Well developed	Internal	External
III	Discoidal flattened	11 cm	9 cm	Brown	Well developed	Internal	External

Table 3. Characteristics of the specimens.

Pregnant Torpedo	Capture month	Total lenght	Weight	Total n. of embryos	Embryo shape	Embryo size	Yolk sac
1	Mid-June	25 cm	1 kg	8	Flattened	2.7-3.5 cm	External
2	End of June	30 cm	1.5 kg	12	Elongated	2 cm	External
3	Early July	36 cm	2 kg	9	Flattened	3.7 cm	External
4	Mid-July	50 cm	2.5 kg	12	Flattened	3.8-4.8 cm	External
5	October	35 cm	1.6 kg	7	Flattened	6-8 cm	External
6	October	50 cm	2.8 kg	14	Flattened	11 cm	Resorbed
Adult Torpedo	**Capture month**	**Total lenght**	**Weight**				
1	Mid-June	20 cm	800 g				
2	Mid-June	20 cm	800 g				

(imprints). Spleen imprints were stained with the May Grünwald-Giemsa stain (MGG) and by the benzidine reaction for hemoglobin. Sections of the paraffin-embebbed tissue blocks were cut at a thickness of 4-10 µm in series, each consisting of four adjacent sections: the first was processed for CD34, the second for CD38 immunoreactivity, the third for negative control and the fourth was stained with the Galgano trichromic method or hemalum-eosin. Subsequently, double immunocyto-chemical reaction was performed on other sections of stage III embryos.

Single immunocytochemistry

The high temperature antigen unmasking technique was performed using citrate buffer and a microwave oven (MW 310 DeLonghi, Treviso, Italy). The sections, mounted on slides, were incubated in 10 mM citrate buffer, pH 6.0, in capped plastic jars, placed in water and microwaved at 400 W (2 treatments for 5 min); the slides were then kept in citrate buffer at room temperature for 20 min. After washing in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), the sections were treated for 15 min in 3% H_2O_2 to inactivate endogenous peroxidase activity and incubated for 60 min at room temperature in 5% normal horse serum (NHS; Dako, Glostrup, Denmark) in PBS containing 0.1% Triton X-100 (Sigma). The sections were then incubated overnight, in a humid chamber at 4°C, with mouse mono-clonal antibodies that recognize CD34. Three different clones of anti-CD34 anti-bodies were used (DAKO, clone BI3C5; Novocastra, clone Qbend10; Boehringer, clone TÜK3), all diluted 1:25 in NHS. Consecutive sections were incubated with mouse monoclonal anti-CD38 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:50). After several rins-es, the sections were incubated for 2 h in biotinylated goat-anti mouse immunoglobu-lin G (Vector Laboratories, Burlingame, CA, USA), diluted 1:50 in NHS, followed by incubation for 1h at room temperature in avidin-biotin-peroxidase solution (ABC Kit, Vectastain, Vector) in PBS, and then for 10 min in 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 in 0.01 M Tris-HCl-buffered saline, pH 7.6. The sections were finally counterstained with hemalum for 30 s and some of them were also counterstained with eosin for the demonstration of eosinophilic myeloid cells.

Imprints of newborn rat bone marrow were used as positive controls. As negative controls, some sections were processed with the same protocol omitting the primary anti-body; no immunostaining was detected in these preparations. The clones of anti-CD34 antibodies that resulted in the most effective immunostaining were BI3C5 on paraffin sections and TÜK3 on touch preparations, respectively.

Double immunocytochemistry

After the antigen unmasking technique two types of double immunolabelling were performed on spleen sections of stage III embryos. Using polyclonal rabbit anti-human CD38 antibody (Santa Cruz Biotechnology; dilution 1:50) as primary antibody; goat anti-rabbit antibody (Santa Cruz Biotechnology; dilution 1:25)-alkaline phosphatase-conjugate (ALP) was used as secondary antibody and then visualized through ALP Vector Red Alkaline Phosphate Substrate Kit I (Sk-5100, Vector Labs, Burlingame, CA, USA) after washing in TRIS-HCl buffer (0.1M pH 8.4 and incu-bation with levamisole as endogenous ALP inhibitor (SP-5000 Levamisole solution; Vector Labs). The second immunolabeling was performed by using monoclonal anti-CD34 antibody (clone BI3C5, Dako) with the same procedure used for single immunocytochemistry (ABC-DAB reveal-ing method) and alternatively, by using pro-tein A linked to colloidal gold (20 nm) as secondary antibody, that was evidenced by silver-enhancer method (Kit Vector Labs).

Quantitative evaluation

A quantitatitive evaluation was per-formed only on spleen sections of stage III embryos, because no immunoreactivity was observed in the previous stages. The spleen sections of three specimens of stage III were analyzed.

The single immunostained spleen cells of the white pulp were counted in 4 adjacent sections per each of the two CD marker immunostaining. The counting was per-formed on 4 slides, each containing 4 full-thickness spleen sections, per each speci-men from stage III embryos. The relative proportion of CD-immunopositive or immunonegative cells was evaluated, counting the immunostained splenic cells and comparing them with the total number of lymphoid cells in the white pulp.

Densitometric evaluation of the intensi-ty of immunostaining of each marker in the splenic white pulp was performed with image analysis of consecutive sections treated with single immunoreaction. A sam-ple of 200 immunostained spleen cells per marker (CD34 or CD38), with nuclei (unstained or lightly stained) in the focal plane, were randomly selected. The cells were characterized on the basis of their morphological features, as described in a previous study[9] as lymphoid progenitor cells in various maturative stages *i.e.*, pro-lymphocytes (preB cells) and lymphocytes. Images were acquired, under constant

light illumination and at the same magnifi-cation, using the digital camera Kontron Elektronik Progress 3008 connected to the microscope and the image analysis software Kontron Elektronik Imaging Computer System KS300 V2.0 (Zeiss, Milan, Italy). In each section the zero value of optical density (OD) was assigned to the back-ground, *i.e.*, a portion of the spleen devoid of stained cells. For statistical analysis of densitometric data, the inter-group differ-ences in the average OD values were evalu-ated with one-way analysis of variance (ANOVA) followed by the *post-hoc* Bonferroni test.

Results

Developing and mature spleen morphology

Embryos were classified as stages I, II, III according to their structural presentation. Early torpedo embryos were elongated in shape and during later embryonic stages the body gradually flattened out. In the uterus of pregnant torpedoes, embryos of different sizes coexisted, all at the same stage of embryonic development. The characteris-tics of the analyzed embryonic stages are summarized in Table 2.

Stage I

In the sagittal section through the embryo wholemount, the spleen was observed to be located posterior to the esophagus, anterior to the bowel, surmount-ed dorsally by the liver. The spleen dis-played an elongated shape along the rostro-caudal axis and measured 0.7 x 0.2 mm (Figure 1A).

In sections stained with the trichromic method, the spleen appeared encased in a thin capsule of connective tissue that entered the splenic parenchyma to form a thin network containing a dense aggregate of blastic cells. Some of these elements were represented by lymphoid-like cells ensheathing blood vessels within which numerous mitotic cells were identifiable. Large erythroid cells were the only cells observed in the vascular lumina (Figure 1B).

Stage II

In the mid-sagittal sections stained with the trichromic method, the spleen was 1.3 x 0.6 mm in size, surmounted by the pancreas and displayed a more rounded shape than in stage I. The fresh preparations of the tissue were light brown in color due the onset of appearance of red pulp. The parenchyma showed a great number of blood vessels and large sinuses, and was encased by a thin
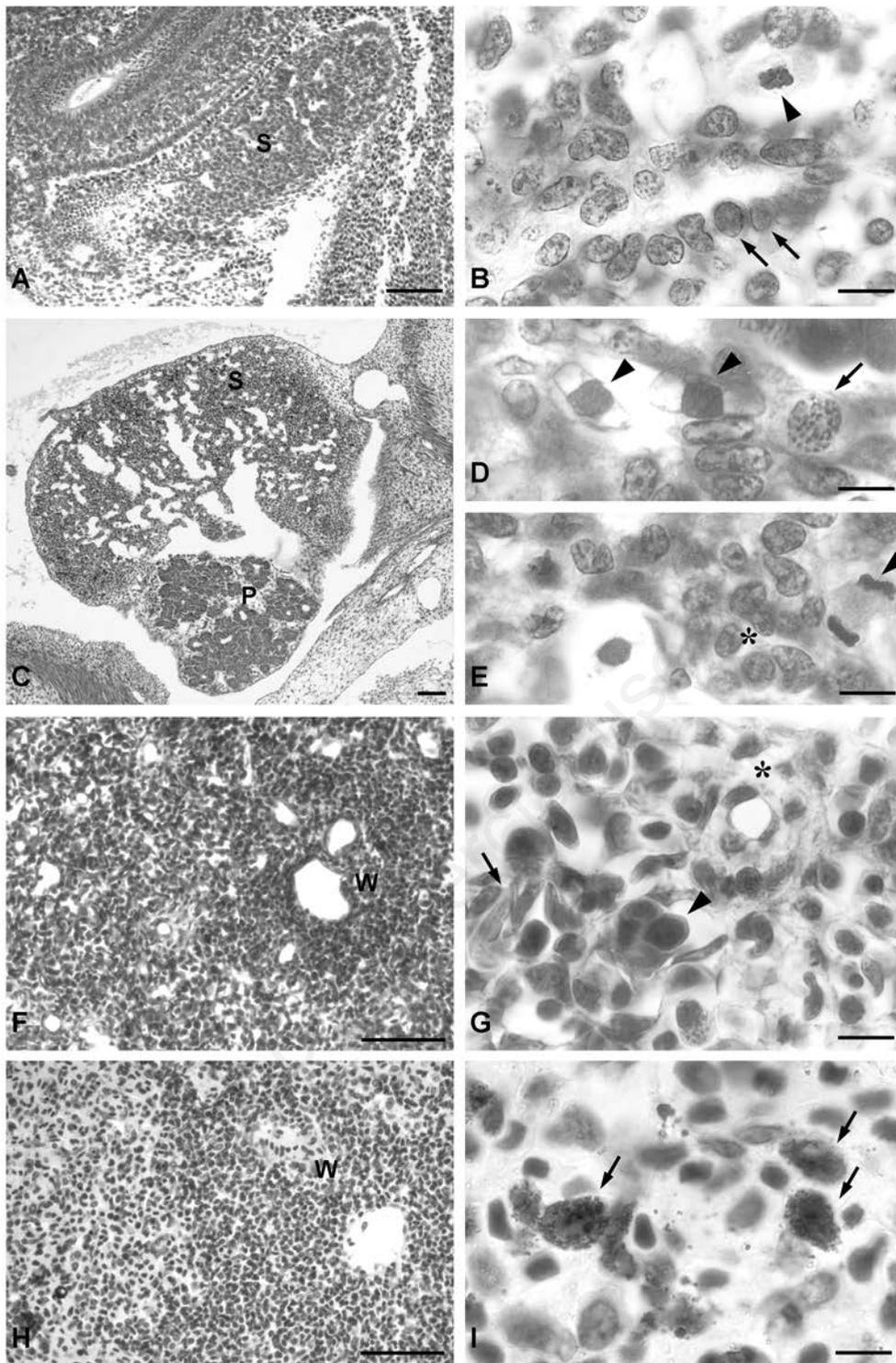
Figure 1. A) Sagittal paraffin-embedded section of embryonic stage I showing the elongated spleen (S), measuring 0.7 × 0.2 mm, near the oesophagus; Galgano's trichromic stain; scale bar: 100 μm. B) Detail of panel A to show an erythroid cell in a blood vessel (arrowheads) and two lymphoid-like cells that start organizing around the blood vessel; scale bar: 13 μm. C) Mid-sagittal section of embryonic stage II showing the spleen (S), 1.3 × 0.6 mm in size, surmounting the pancreas (P); note the increase of large sinuses and blood vessels; scale bar: 200 μm. D,E) Details of panel C to show initial erythropoiesis: erythroid cell in initial mitosis (arrow) in panel D and final mitosis in panel E (arrowhead); periarterial lymphoid sheaths that start organizing (asterisk); scale bar: 13 μm. F) Transverse section of a spleen of embryonic stage III; the spleen measures 5 × 1.3 mm and displays a well-defined white pulp (W) with lymphoid cells at various stages of maturation; Galgano's trichromic stain; acale bar: 100 μm. G) Detail of panel F to show an ellipsoid (asterisk), an early hemopoietic stem cell (CD34+) (arrowhead) and erythopoiesis in the red pulp (arrow); scale bar: 13 μm. H) Transverse section of the spleen of an adult torpedo showing the white pulp (W) and a structure very similar to that of stage III in panel F; Galgano's trichromic stain; scale bar: 100 μm. I) Detail of panel H to show melanomacrophages (arrows), which appear in the parenchyma only after birth; scale bar: 13 μm.

capsule penetrating the parenchyma to form a thin weft (Figure 1C). Red pulp formation started at this stage and its blood vessels contained only large erythroid cells (Figure 1D). In addition, the organization of periarterial lymphoid sheaths starts (Figure 1E), and structures similar to the ellipsoids of the adult spleen were also observed. Several cells in mitosis were present (Figure 1 D,E).

Stage III

At this stage the spleen could be easily distinguished in the abdomen. The organ was 5 × 1.3 mm in size and had an intense red color in fresh preparations. Its histological structure was very similar to that of the adult torpedo containing a well-defined white pulp with lymphoid cells at various levels of differentiation (lymphoblasts, prolymphocytes, plasma cells) and some eosinophilic myeloid cells (Figure 1F). Erythroblasts and erythrocytes were located in the intravascular regions of the red pulp (Figure 1G).

Adult

The spleen of the adult animal (800 g in weight) was 2.5 × 1.5 cm × 0.5 cm in size and intensely red in fresh preparations. Transverse sections of the organ stained by the trichromic technique presented a voluminous spongy vascular matrix (the red pulp), in which the more compact lymphatic white pulp was embebbed. The parenchyma displayed a thin web of connective tissue (Figure 1H). The white pulp contained small lymphocytes and their precursor cells, plasma cells and some myeloid cells. Mature erythrocytes were very numerous in the red pulp and erythroblasts and thrombocytes at various stages of maturation were also present. Melanomacrophages (not evident at the examined embryonic stages) were also evident in the splenic parenchyma (Figure 1I).

In MGG-stained spleen imprints, rare cells exhibited the following characteristics: a round shape, with an average diameter of 15-18 μm and a high nucleus/cytoplasm ratio, an intensely basophilic cytoplasm and a nucleus containing reticular chromatin and an evident nucleolus (Figure 2G).

Immunocytochemical findings

Microwave treatment permits an amplified antigen labeling and significantly reduces intensity of the background of tissue sections. This procedure proved to be very effective in the unmasking of CD34/CD38 antigen. The immunodetection of the CD34 antigen proved to be most successful in this study with the use of clone B13C5. Other clones gave lesser immunoreactivity. At embryonic stages I and II, no CD34 immunoreactive (ir) cells were observed in the spleen, except for some of the circulating large erythroid cells and endothelial cells of the blood vessel walls. At embryonic stage III, numerous CD34ir cells were seen in the isolated spleen, and they exhibited various intensities of immunostaining. The most intensely immunostained cells were the lymphoid-like cells with a large, immature nucleus. They were CD38 negative, as demonstrated by the findings observed in the adjacent sections (see Figure 2 A-D for stage III embryo spleen, and Figure 2 E-H for the adult spleen). At stage III, CD34 immunoreactivity of various intensity was detected in cells of the myeloid and lymphoid lineages at different stages of maturation, as well as in the large immature erythroid cells and in the endothelial cells of blood vessel walls. CD34 immunoreactivity progressively decreased, until it disappeared, at maturation. CD38 immunoreactivity was detecteable in lymphoid and some myeloid committed cells, but it decreased in the next maturative stages of lymphoid cells and disappeared at maturity.

Therefore, the cell population labeled with anti-CD34 antibodies, at stage III, was very heterogeneous. The most immature cells were intensely immunostained, while a less intense CD34 immunoreactivity was displayed by the committed cells, and finally the well differentiated cells, such as lymphoid or myeloid cells, showed a weak or no immunostaining. In brief, at stage III, while there is a progressive decrease in CD34 immunoreactivity, a CD38 immunoreactivity appears in lymphoid progenitor cells in the splenic white pulp (Table 2; Figure 2 A-D). The subsequent lymphoid maturational stages displayed a decrease of both CD34ir and CD38ir cells. CD34neg/CD38ir cells of the white pulp appeared to be prolymphocytes (preB cells) (Figure 2B) and CD34neg/CD38neg cells were lymphocytes.

In the adult spleen, CD34ir/CD38neg cells were rare (1 per section in 30% of the sections) and displayed a shape with a high nucleus/cytoplasm ratio, an immature nucleus with an evident nucleolus (Figure 2 E,F,H). They were identical to those detected in stage III embryos (Figure 2 A-D). Double immunolabelling confirmed the existence of the four populations mentioned above, by displaying only ALP-positive cells (CD34-/CD38+) (Figure 3 B,D), ALP and DAB positive cells or ALP and silver positive cells (CD34+/CD38+) (Figure 3 E,B, respectively), negative cells (CD34-/CD38-) such as lymphocytes (Figure 3 B,D,E) and only DAB positive or silver positive cells (CD34+/CD38-) which are very rare (Figure 3 B,D, respectively). Lymphocytes have been observed more often near the wall of blood vessels, sometimes appearing to enter the blood vessels (Figure 3E).

Image analysis data

Four immunophenotypes were observed in stage III embryos and their classification was based on the mammalian counterparts: i) CD34+/CD38+ cells (lymphoid progenitor cells, such as lymphoblasts; ii) CD34-/CD38+ cells (prolymphocytes-pre B cells), and iii) CD34-/CD38- cells (lymphocytes). Moreover, rare CD34+/CD38- round cells were observed in the peripheral zone of the white pulp. These cells were round, displayed a high nucleus/cytoplasm ratio, an immature central nucleus and a nucleolus. They were consequently assumed to represent HSCs because they showed the characteristic morphologic features and phenotype of this cell type.

The white pulp of the spleen of stage III embryo, the tissue upon which this study was centered, exhibited lymphocytes of all levels of maturation as well as some immature myeloid cells. Quantitative analysis of the frequency of the cells in in this locus confirmed that HSCs were rare (1±0.3%, expressed as the average from the three specimens ± standard deviation of the mean). The most frequent cell types were lymphoid progenitor cells (58.2±6.5%), followed by preB cells (24.8±4.2%) and by lymphocytes (16.5±5.1%) (Figure 4B).

The densitometric analysis of the immunostaining intensity confirmed the morphological observations. The most marked CD34 expression (38.87±0.69; values are given in OD units value ± SEM) occurred in rare cells (about 1% of the sampled cells which were CD38-negative in the consecutive section) (Figure 4A). These cells were located in the peripheral zone of the white pulp, between the white and red pulp. The majority of the other cells exhibited progressively decreasing OD values down to the lowest OD value (equal to the background) in the most differentiated lymphoid cells. CD38 immunoreactivity was highest in lymphoid progenitor cells (28.07±1.6) in which CD34 immunostaining was decreased (25.79±5.18) with respect to its maximum. The reactivity decreased further (16.35±1.5) in the more differentiated lymphoid cells which exhibited CD38 positivity, but no CD34 positivity (Figures 2 A,B and 4A). These cells appeared to be prolymphocytes (preB cells). The most mature lymphoid cells, the lymphocytes, appeared to be CD34 and CD38 negative (Figures 2 A,B and 3 B,D,E). Statistical evaluation indicated that the difference in the staining intensity of the different populations of CD-immunoreactive
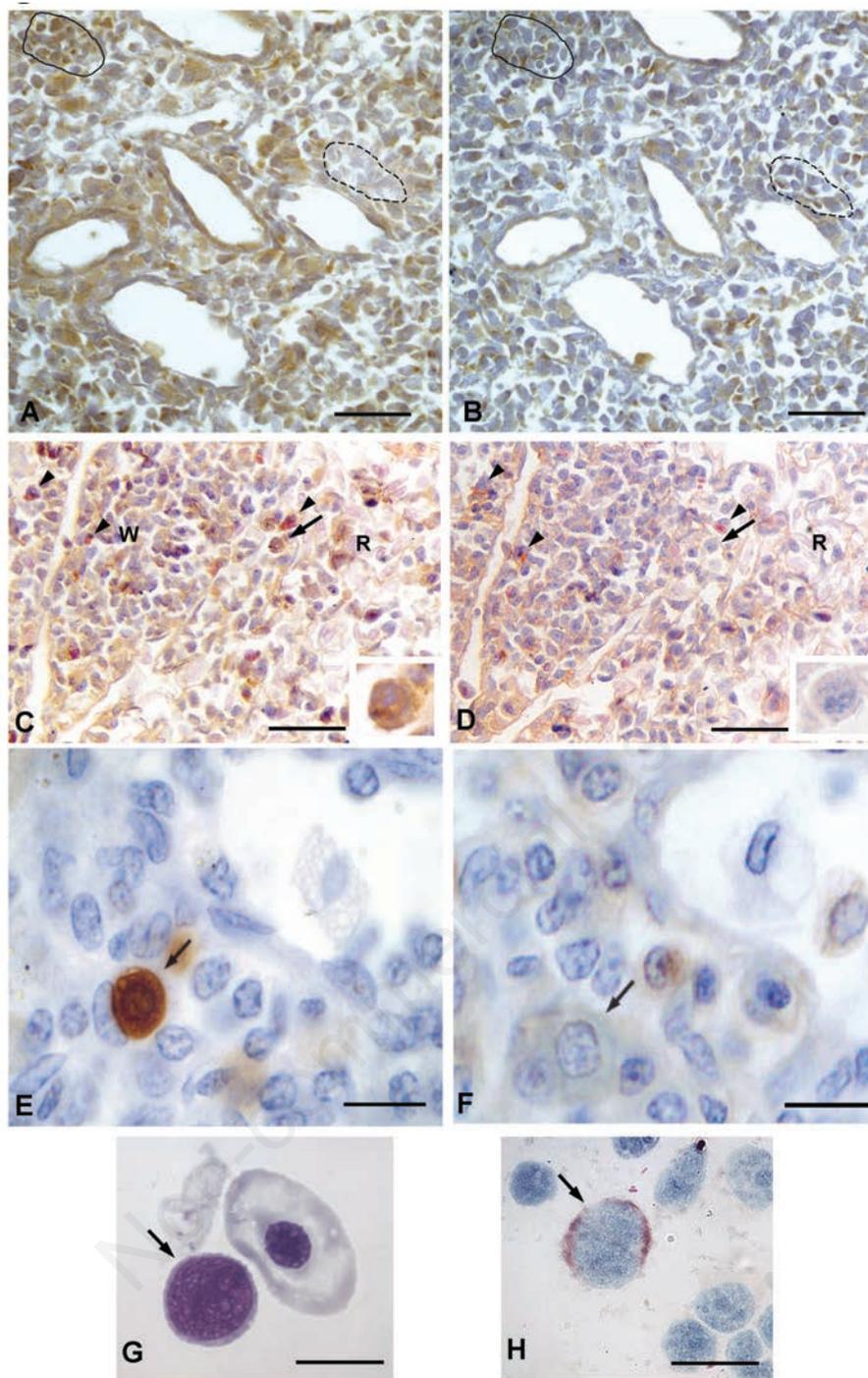
Figure 2. Immunostaining of consecutive sections from an embryonic spleen of stage III (A-D) and an adult spleen (E,F) with anti-CD34 (A,C) and anti-CD38 (B,D) counterstained with hemalum or hemalum-eosin (C,D) and of consecutive sections from adult specimen spleen (E,F) and from spleen imprint (G,H) with anti-CD34 (E,H) and anti-CD38 (F) counterstained with hemalum. A) White pulp in which lymphoid cells, arranged around arteries, exhibit CD34 immunostaining (brown stained) at various intensity; some lymphoid cells, probable prolymphocytes (preB cells) display no CD34 immunostaining (cells in the dotted-line circle); CD34+ lymphoid cells which are CD38+ in consecutive section (2B) are circled with entire line. Endothelial cells of the vessel walls are CD34 immunostained. B) Some of the lymphoid cells which are CD34 immunostained (in A) exhibit CD38 immunostaining at various intensity (in entire line circle); probable prolymphocytes (in dotted line circle) are only CD38 immunostained. Endothelial cells of vessel walls display no CD38 immunostaining; scale bar: 50 μm. C,D) Consecutive sections (4 μm thick) of white pulp (W) and red pulp (R) counterstained with hemalum-eosin; note in 2C the round immature CD34+ cell located in the peripheral zone of the white pulp near the red pulp (black arrow); this CD34+ cell (enlarged in the box) appears to be CD38- in consecutive section (D, black arrow) (enlarged in the box); eosin was used to demonstrate the occurrence of eosinophilic myeloid cells (black arrowheads), which infiltrate white pulp; scale bar: 50 μm. E,F) Consecutive paraffin sections of adult spleen in which the round cell intensely CD34 immunostained (arrow) display no CD38 immunostaining in the consecutive section (2 h) (arrow); scale bar: 15 μm. G,H) Spleen imprints (touch preparations) of adult torpedoes. May Grünwald Giemsa stain in G: a hemopoietic stem cell with intensely basophilic cytoplasm (arrow). CD34 immunostaining in a hemopoietic stem cell in H (arrow); the cytoplasm shows an intense brown-stained immunoreactivity; scale bar: 18 μm.

cells was highly significant (one-way ANOVA, for CD34, $F_{(18.306)} = 389.861$; P<0.0001 and for CD38, $F_{(2.633)} = 2573.162$; P<0.0001). The post-hoc comparison of both CDs intensity of immunostaining, indicated statistically significant (Bonferroni test, P<0.01) between the different cell populations. No significant difference was observed between the intensity of CD38 staining of HSCs and lymphocytes (Figure 4A).

Thus, the comparison with the densitometric data indicated that CD34 staining decreased through cell maturation and CD38 staining was expressed at its highest levels in lymphoid progenitor cells and decreased through maturation (Figure 4).

Discussion

Many markers currently used to recognize HSCs in adult are not applicable to HSCs in embryos. Moreover, surface marker expression on HSCs varies during development as well as between strains and species.[22,23] It can also be held that CD34+ cells are the HSCs of the adult and that they appear during embryonic development when the definitive hemopoietic organs are being established. With regard to the embryonic stages of torpedoes, stage I corresponds to stage 28 in *Squalus acanthias* as described by Scammon.[24] At this stage torpedo embryos have not yet acquired the flattened shape, in agreement with the observations of Ranzi[25] in *Torpedo marmorata*.
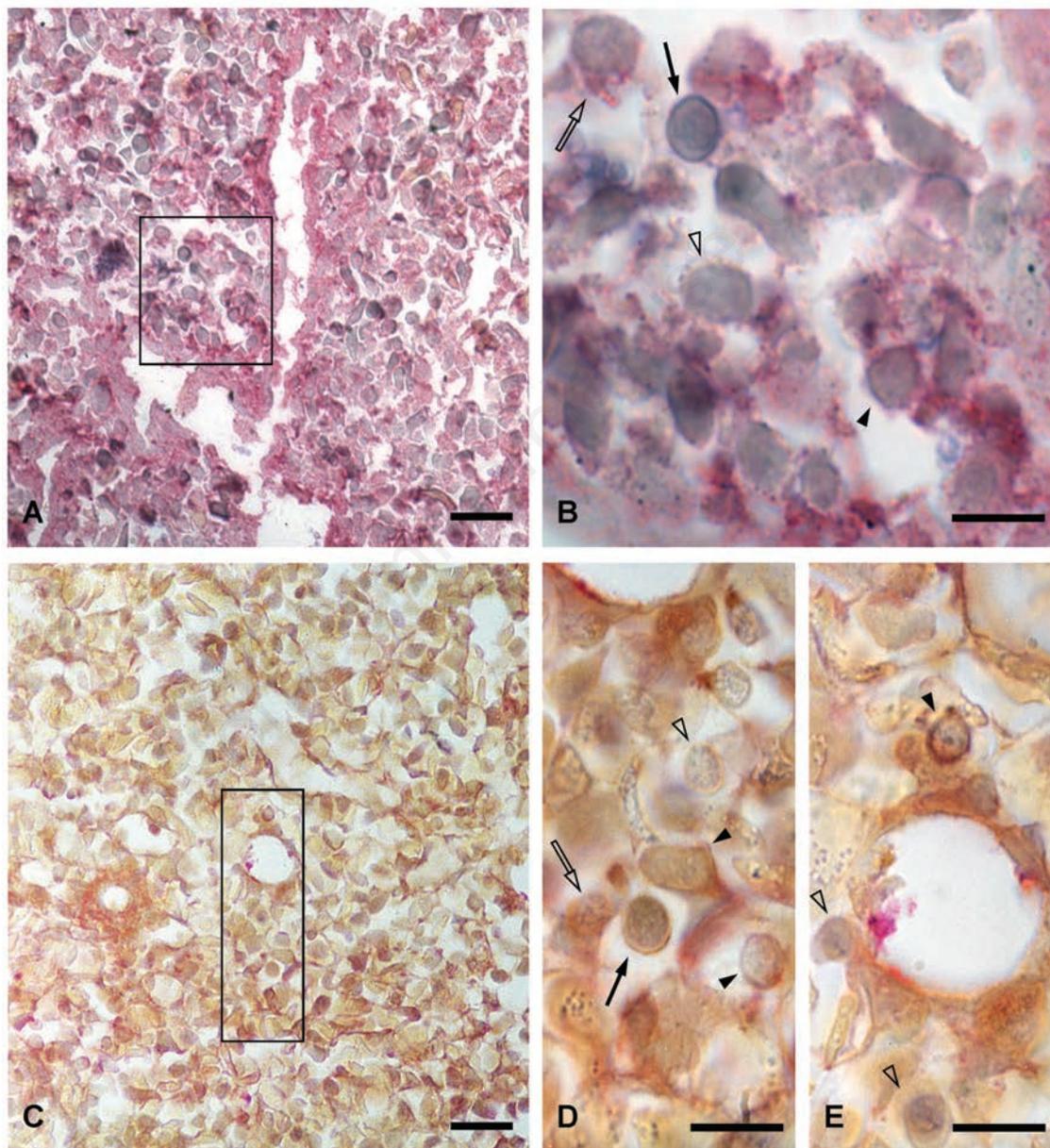
The gross structural configuration of the

Figure 3. Double immunostaining of sections from an embryonic spleen of stage III. Four cell populations can be observed: CD34+/CD38- cells (black arrow), CD34+/CD38+ cells (black arrowhead), CD34-/CD38+ cells (open arrow), CD34-/CD38- cells (open arrowheads). A,B) CD34-Silver/CD38-ALP double immunostaining; the boxed cells in A are doubled in B. C,D) CD34-HRP-DAB/CD38-ALP double immunostaining; the boxed cells in A are doubled in D and E. E) Lymphocytes have more often been observed near the wall of blood vessels sometimes appearing to enter the blood vessels (open arrowheads). Scale bars: A,C) 30 μm; B,D,E) 35 μm.

stage II torpedo, on the other hand, is not comparable to any of the stages described by Scammon[24] for the squalus as the torpedo presents the typical flattened shape of rays. Ranzi[25] reported only the weight of *Torpedo marmorata* at the equivalent embryo stage II level of development (14 g). During embryonic development, two main structural changes occur in the torpedo spleen before the organ attains its final structure with a red and a white pulp, which are distinguishable on the basis of their different cell composition. In agreement with published data concerning other fish species, the present investigation indicates that in torpedoes, earliest anlage of the spleen consists of mesenchymal tissue and a population of lymphoid-like cells. The progenitors of the erythroid, lymphoid and thrombocytic lineages are evident only after the formation of large sinuses and vessels (stage III). Our findings have also pointed out that the macroscopic and microscopic structure of the spleen in stage III embryos is very similar to that of the adult animal, and that only in stage III embryos some splenic cells exhibit the CD34 and CD38 phenotypes. In particular, single immunostaining on adjacent sections of the torpedo's spleen showed co-expression of the two clusters of differentiation in some cells. This co-expression was confirmed by double immunostaining. These cells are lymphoid-committed stem cells, located in the white pulp, in which CD34 expression was down regulated, while CD38 expression appeared. It is well known that 99% of CD34+ cells in mammalian bone marrow also display the later-appearing CD38 antigen, which is a differentiation marker of the lymphoid lineage[26] while the expression of CD34 reaches its maximum on the earliest progenitors and decreases progressively during maturation. The most immature mammalian B-lymphoid precursor, known so far, is CD34+.[27]

The present finding in the torpedo spleen of rare CD34+/CD38- cells exhibiting immature cell features, provides a strong indication that these cells are HSCs. Moreover, the detection of CD34 immunostaining in the large immature erythroid cells and endothelial cells, and their CD38 negativity, are consistent with the phenotype of their counterparts in mammals. Moreover, it has been ascertained that the hemangioblast is the common precursor for endothelial and hematopoietic cells[28] and therefore it is not surprising that endothelial cells reveal CD34 immunoreactivity (Figure 2E). That is, CD34 is a marker for early hemopoietic and endothelial cell lineages, which are believed to derive from a common precursor, and CD38 is a lymphoid lineage marker.[29,30]

It should also be considered that, in the present study, positive controls of newborn rat bone marrow exhibited cells morphologically similar to those of torpedoes, exhibiting a CD34+/CD38- phenotype.

The identification of CD34 antigen was realized with anti-rabbit and anti-mouse antibody in other studies.[18-20] It can be hypothesized that part of antigen sequence is conserved during evolution. The cross reactivity of several antibodies against human and murine cell surface receptors was studied by Cook *et al*.[31] in leukocytes of snapper (*Pagrus auratus*). Antibody A452, against the intracytoplasmic tail of the epsilon chain (ε) of the T cell receptor-associated CD3 complex (CD3 ε), binds to an intracellular epitope on a sub-population of snapper IgM peripheral blood leukocytes. The cross reactivity of A452 was found in a number of different species including monkey, pig, horse and cow. This suggests that this peptide is highly conserved across a diverse range of vertebrates and is bound to a subpopulation of peripheral blood leukocytes. Previous studies on torpedos have also demonstrated a similarity of torpedo and mammalian lymphocytes, based on their cytochemical features and presentation of immunoglobulins as revealed via polyclonal antihuman antibodies anti-IgM, anti-IgG and anti-IgA.[32-35]
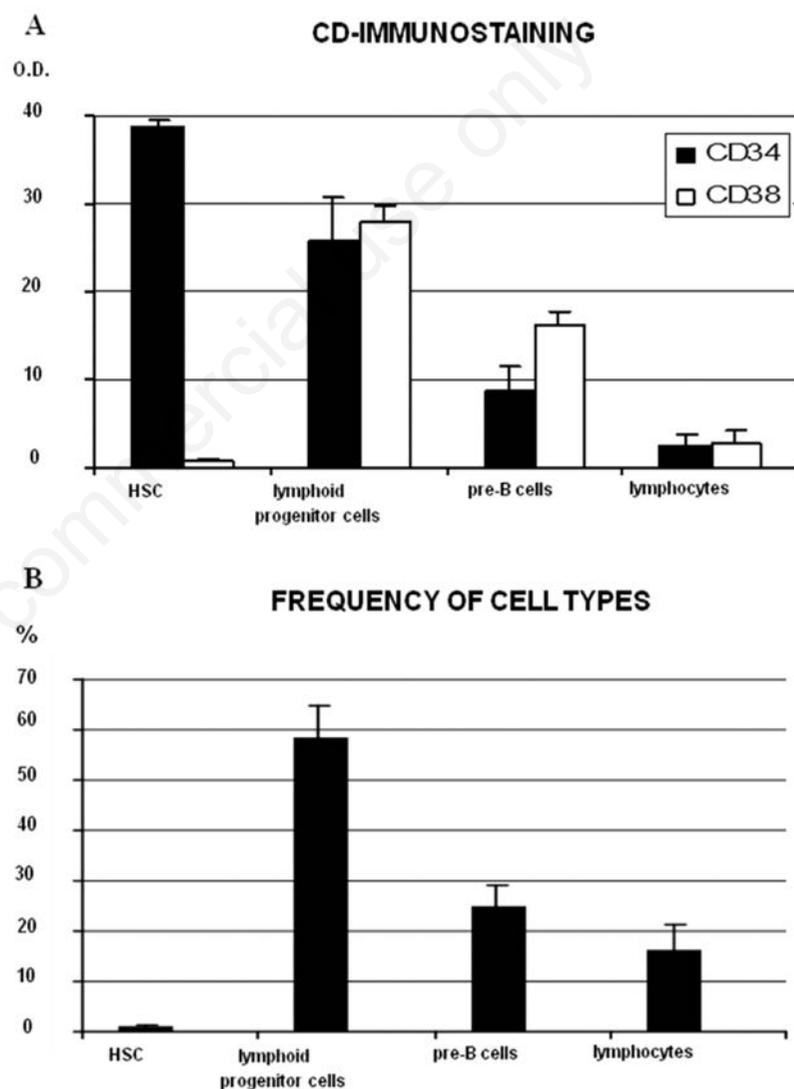

Figure 4. A) Histogram of the intensity of CD immunostaining, measured in optical density (O.D.) units, in the white pulp cells of torpedo spleen; values (numerical values are specified in the text) are expressed as average of 3 animals per group ± standard deviation of the mean. B) Histogram of the relative proportion of white pulp cells of torpedo spleen; values (numerical values are specified in the text) are expressed as average of 3 animals per group ± standard deviation of the mean.

According to contemporary tenets, cartilaginous fishes are considered to be the most primitive vertebrates with immunoglobulin (Ig).[36-39] More recently, Flajnik[40] has proposed that elasmobranchs are the oldest group of vertebrates with an adaptive immune system based on Ig/TCR/MHC. They have been shown to have not only IgM but at least two other isotypes, IgNAR and IgW. The occurrence of IgM and TCR genes was studied also in other elasmobranchs, such as *Raja eglanteria* and *Ginglymostoma cirratum*.[41,42] Two classes of immunoglobulins 7S IgM and IgNAR have been discovered in unfertilized nurse shark eggs and in the yolk stored in external and internal yolk sacs of developing embryos. These data suggest that 7S IgM and IgNAR are selectively transported into oocytes, relative to other serum proteins.[43] An investigation of the lymphocyte subpopulations in the teleostean fish, *Diplodus sargus,* has documented its maintenance of two subpopulations of lymphocytes (B and T) on the basis of their immunostaining with antihuman CD2, CD3, CD4, CD8 antibodies.[44] This information lends support for the premise that the current finding of the CD34 and CD38 phenotype of torpedo lymphoid cells is consistent with that of the corresponding mammalian cells.

In our study, the absence of CD34+ cells in early embryos (stages I and II) might be due to the incomplete development of the spleen microenvironment, which might not yet be able to trigger hemopoiesis or to be colonized by blood stem cells or to activate this marker on stem cells. In addition, the torpedo embryonic spleen could contain quiescent stem cells which do not express CD34 but give rise to CD34+ cells, as described in mammals.[45] CD34 is actually an activation marker on HSCs and a direct adhesion mediator to stroma of hemopoietic tissues.[46] The identification of the first hormone or growth factor that starts stem cells moving is still a matter of study, since their activation depends upon specific environmental circumstances and growth stimuli.[26]

In elasmobranchs, hemopoiesis starts with the development of primitive nucleated erythroid cells in the yolk sac and in a distinct dorsal-lateral compartment of the embryo, known as the intercellular mass of Oellacher (described in 1872), as reported by Zon.[2,47] Our investigation shows that in torpedoes, this is followed by the appearance of CD34+/CD38- precursors of definitive hemopoietic lineages in the spleen during embryonic stage III. A study of HSCs in the mouse embryo has suggested two independent origins of HSCs during development, namely from a transient system in the yolk sac and from the definitive system in the aorta-gonad mesonephros region.[48] A yolk-sac origin of definitive hemopoiesis in the avian embryo had been ruled out by the experimental investigation of Dieterlen-Lievre.[4]

The relationship of primitive erythroid cells with the other hemopoietic populations in elasmobranchs is still unknown. It remains to be determined whether early torpedo HSCs migrate from yolk sac blood islands and colonize hemopoietic tissues or whether they have an intraembryonic origin.

Our study pointed out that the speen of stage II torpedo embryo is characterized by the development of a vascular bed without detectable hemopoiesis. Consequently, no CD34 precursors were observed before the onset of hemopoiesis. The present results indicate that numerous cells express detectable levels of CD34 antigen in the spleen only at stage III. Most of these cells which exhibited less than maximal levels of CD34 antigen along with relatively higher levels of the CD38 antigen, were localized in white pulp of the spleen and are recognized as early progenitors of the lymphoid cells. Only rare cells displayed the CD34+/CD38- phenotype and can be considered the pure HSCs, the same markers that identify this cell in mammals. In the mature torpedo spleen, pure HSCs are very rare. Although it is well known that definitive hematopoiesis in vertebrates later involves HSCs, which occur in the aorta-gonad-mesonephros (AGM) region of the developing embryo,[50] very likely, the CD34 immunoreactive HSCs are even more later than the primitive HSCs and appear in the developing spleen and in the lymphomieloid tissues (*data not shown* of a research in progress) of torpedo embryos.

Experiments performed in the different mammalian and non-mammalian animal models have documented that HSCs appear at one site and rapidly colonize other loci via the blood circulation. In particular, the autohemotransplantation performed in sublethally X-ray-treated torpedoes has demonstrated the capacity of circulating blood to recolonize hemopoietic tissues.[8]

In brief, *Torpedo* embryo is an excellent model for the detection of the emergence of definitive HSCs. HSC presentation is a relatively late event and occurs in the definitive spleen. The spleen of bone-marrowless vertebrates is likely to be a blood–forming intraembryonic organ, in which the cell microenvironment supports hemopoiesis and persists as the main hemopoietic organ throughout life in all elasmobranchs. Our study indicates that the torpedo's CD34+ HSCs share cytoplasmic and nuclear characteristics, as well as phenotypic expression, with their mammalian counterparts. In addition, the results of the present study demonstrate the evolutionary conservation of the CD34 marker in the elasmobranchs.

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