Perichondrial Chondrogenesis and Periosteal Osteogenesis by Localized Graft-vs-Host Reaction in Mice

(GvH reaction / perichondrium / periosteum / mice)

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Abstract. Localized GvH reaction produced by the subcutaneous injection of parental lymphoid cells into the ear lobe of F_1 recipient mice results in local activation of perichondrial chondrogenesis. Similarly, when the GvH reaction was elicited in shanks, the periosteal membranes at the site of immune reaction were stimulated to proliferate and to produce new bone. However, syngeneic lymphocytes activated *in vivo* by Con A and untreated syngeneic spleen and bone marrow cells administered locally produced similar response of bone and cartilage. Thus the lymphocytes activated either in the course of GvH reaction or syngeneic lymphocytes grafted into heterotopic sites presumably release mediators capable to stimulate periosteal/perichondrial membranes.

Mitogen-activated immune cells, when grafted into syngeneic recipient mice, activate local periosteal and perichondrial membranes to proliferate and to produce new bone and cartilage (Włodarski, 1991). Similarly, subcutaneous administration of various immunomodulators (concavalin A (ConA), phytohaemagglutinine (PHA), Carrageenan IV), which elicit a local inflammatory reaction, resulted in activation of periosteal/perichondrial membranes in adjacent skeletal tissues (Włodarski and Galus, 1992). Such stimulation mimics, to a lesser degree, the periosteal/perichondreal activation by Moloney sarcoma virus-induced tumours and by other tumours of non-viral history (Włodarski, 1987, 1989; Włodarski and Reddi, 1987; Włodarski et al. 1979, 1993, 1994, 1995).

It was postulated that tumour and lectin-stimulated lymphocytes release growth factors capable of activating osteoblastic/chondroblastic cell proliferation (Włodarski and Galus, 1992). There is enough evidence of the effects of immune cell products (interleukin 1 (IL-1), tumour necrosis factor (TNF)) on bone proliferation and resorption (Goven et al., 1985; Pfeilschifter et al., 1989). The aim of the present work was to examine whether local graft-versus-host (GvH) reaction in an F_1 hybrid model would trigger periosteal/perichondrial membrane activation at the reaction site.

Material and Methods

Animals

The BALB/c, CBA/H, CFW/L1 and C57Bl mice were bred for three generations in our animal facilities before being used in experiments or for production of F_1 hybrids. Two to three months old animals of both sexes were used.

Histocompatibility antigens of the CFW/L1 inbred strain have not been determined (W. Lasek, personal communication).

Induction of the local GvH reaction

The test for local GvH reaction was performed according to Tessenov (1976). Recipient F_1 mice (BALB/c × C57Bl) and (CBA/H × BALB/c) were injected with parental popliteal lymph node cells, femoral bone marrow cells or with spleen cells. In both combinations mice differ in MHC plus multiple non-MHC antigens (C57Bl mice are $H2^b$, CBA/H are $H2^k$ and BALB/c are $H2^d$); thus both strain combinations represent a strong (and comparable) genetic barrier. To examine the effect of localized GvH reaction on chondrogenesis, the parental cells were injected subcutaneously into the pinna. To evaluate the effect of GvH reaction on osteogenesis, the parental cells were injected into the right shank muscles, whilst the left (contralateral) shank was injected with saline only.

Preparation of cells

Popliteal lymph nodes of the parental strain were cleaned from the adjacent connective tissue, minced with scissors in RPMI 1640 serum-free medium, sieved through a metal 000 mesh, counted and sedimented. Ten million cells were suspended in 0.1 ml of RPMI 1640 medium and injected intradermally into pinnas of F_1 hybrids. Bone marrow cells were obtained by flushing out the femoral midshaft cavity with RPMI 1640 serum-free medium and pipetted to obtain a single-cell suspension. Splenocytes were isolated using the same method as

lymph node cells. Twenty million lymph node cells, 20 million bone marrow cells and 50 million spleen cells were suspended in RPMI 1640 and injected subcutaneously into the ear lap (pinna) in a volume not exceeding 0.2 ml. To examine the effect of GvH reaction on osteogenesis, one hundred million spleen cells, suspended in 0.3 ml of RPMI medium, were injected intramuscularly into the right shank.

Morphological studies

To evaluate the local GvH reaction on chondrogenesis, the animals were sacrificed 13–47 days after subcutaneous parental cell administration into the pinna. Pinnas were fixed in Bouin solution, examined histologically on serial 7 μm sections, and stained with haematoxylin-eosin (H-E). To evaluate the GvH reaction on osteogenesis, splenocyte-injected and contralateral (control) hind legs were isolated and hydrolyzed in 0.2 M KOH at 65°C overnight. Subsequently, the soft tissues were removed and bones easily isolated. Tibiae were washed in distilled water and dried overnight at 65°C. Paired bones were weighed with an accuracy of 0.1 mg, the change in shank bone weight was calculated by subtracting the saline-injected (left) tibia + fibula weight from the weight of the right hind limb bone injected with parental splenocytes. The bone gain or loss was expressed as a percentage of the contralateral (control) bone weight. Any difference between paired bones exceeding 3% of the control value was considered as significant. The weight difference represented the bone formation (yield of dry bone mass) or bone resorption (loss of dry bone mass) (Włodarski and Galus, 1992). For histological examination of bones at the site of GvH reaction, the shanks were fixed in Bouin solution, decalcified in 10% formic acid and embedded in paraffin. Serial sections from the middle of the shank were stained with H-E.

Evaluation of the GvH reaction following spleen cell grafting into shank muscles

Both, popliteal lymph nodes involved in the GvH reaction and contralateral controls were removed, cleaned from adherent surrounding tissues, and weighed with an accuracy of 0.1 mg. The spleens were weighed to prove the potency of the GvH reaction induced.

In vivo activation of lymph nodes

In addition to the GvH reaction, the effect of grafting of the syngeneic lymphocytes activated in vivo by ConA on shank bones as well as the effect of unstimulated syngenic spleen and bone marrow cells on pinna's cartilage was evaluated.

Female BALB/c mice were injected into the right foot pad with 0.5 mg of ConA (Calbiochem, La Jolla, CA) dissolved in saline. Four to seven days later popliteal lymph nodes from both legs were excised and weighed, and lymphocytes were mechanically isolated from the ConA-activated pooled lymph nodes, suspended in RPMI 1640 medium and injected subcutaneously or intradermally into the pinnae of female BALB/c mice. The weight of ConA-activated lymph nodes was in the range of 7–12 mg (8.8 ± 1.2 mg) compared with 1.3 ± 0.6 mg of the control lymph nodes. Similar lymph node activation by ConA was achieved in CFW/Ll mice.

Results

Frequently, the local GvH reaction was accompanied by activation of perichodreal chondrogenesis of various magnitude (Table 1). Injection of 20×10^6 parental (BALB/c or C57Bl) lymph node cells into the respective F_1 hybrids evoked a mild perichondral reaction in two out of 28 cases during 18–47 day observation. Injection of 50×10^6 of parental spleen cells into (BALB/c × C57Bl)F_1 hybrids activated within the same observation period both, perichondrial and ectopic chondrogenesis (i.e,. the formation of an island of cartilage at some distance from the orthotopic cartilage) in 6 out of 29 cases. The GvH reaction by injection of 25×10^6 bone marrow cells was most effective in activating chondrogenesis. In nearly half of the cases stimulation of perichondrial (i.e., orthotopic) and ectopic chodrogenesis was noted. Moreover, in 2 out of 48 cases of parental bone marrow cell injection into F_1 hybrids, bone trabeculae were found.

Table 1. The effect of localized GvH reaction by parental cells into F_1 recipients on chondrogenesis in situ

No. of grafted cells	Duration (days)	No. of grafts performed	Stimulation of orthotopic chondrogenesis	Stimulation of ectopic chondrogenesis	Presence of bone
(BALB/c × C57Bl)F₁					
Lymph node 20×10^6	18 – 47	28	2	0	0
Bone marrow 25×10^6	13 – 21	11	4	1	0
	23 – 47	48	12	10	2
Spleen 50×10^6	13 – 36	29	2	6	0
(BALB/c × CBA/H)F₁					
Bone marrow 25×10^6	21	3	2	1	0

Table 2. The effect of localized GvH reaction by parental spleen cells into F_1 hybrids on osteogenesis in shank bones

No. of cells grafted in millions	Duration (days)	No. of grafts performed	No. of osteogenesis activation	Weight (mg) of popliteal lymph node (mean ± SD)	
				Contralateral	GvH reactive
(BALB/c × C57Bl)F_1					
100	13 – 21	11	2	1.3 ± 0.6	4.3 ± 2.3
100	22 – 47	48	14 (2[a])	1.3 ± 0.4	2.4 ± 1.0
(CBA/H × BALB/c)F_1					
100	16	4	1	1.2 ± 0.3	3.7 ± 1.7
100	21 – 26	12	1 (2[a])	1.3 ± 0.4	2.9 ± 1.6

[a]bone resorption

Table 3. The effect of ConA-activated syngenic CFW/Ll lymph node and spleen cells on osteogenesis in the site of grafting

Number of cells grafted in millions	Duration (days)	No. of grafts performed	No. of specimens with	
			Bone formation	Bone resorption
Lymph node 25	7 – 33	44	17 (6% ± 3%)[a]	5
Spleen 60	7 – 33	35	15 (5% ± 2%)	0

[a]in parenthesis bone mass gain ± S.D.

Table 4. The effect of grafting of syngeneic bone marrow or spleen cells on cartilage formation in situ

No. of cells grafted in millions	Duration (days)	No. of grafts performed	No. of specimens with chondrogenesis	
			Perichondrial	Ectopic
Splenocytes 50	10 – 19	20	8	3
Bone marrow 20	8 – 17	16	4	0

Activation of ectopic chondrogenesis was also noted following parental bone marrow cell injection into (BALB/c × CBA/H)F_1 hybrids.

The inflammatory reaction in the pinna, as evaluated by the intensity of lymphocyte infiltration at the time of sacrifice, was mild (few lymphocytes per one microscope field) to moderate (several lymphocytes per field).

The injection of $100 × 10^6$ of parental spleen cells into (BALB/c × C57Bl or CBA/H × BALB/c)F_1 hybrids with a strong histocompatibility barrier evoked a GvH reaction, as established by the area-draining (popliteal) lymph nodes enlargement (Table 2).

Stimulation of periosteal osteogenesis (i.e., increase in net bone ash weight) was seen in some cases within the 2nd–3rd week of GvH reaction. With prolonged duration of GvH reaction, in a proportion of cases the activation of bone resorption (bone mass loss exceeding 3% of contralateral bone mass) was observed. The GvH reac-tion ceased with time, as judged from the popliteal lymph node weight normalization. The weight of spleen in F_1 animals injected with parental spleen cells into shank muscles was within the normal values (99 mg ± 8 for CBA × BALB/c on day 26th, 102 ± 16 for BALB/c × C57Bl on 22nd day), no splenomegaly was observed.

Popliteal lymph node cells, activated *in vivo* by the mitogen ConA given into a foot pad 4–7 days prior to cell harvest, and spleen cells obtained from ConA-treated mice, when grafted into syngeneic CFW/Ll mice, had a dual effect on shank bones (Table 3). In many cases (17/44 and 15/35) bone formation was observed, but in a proportion of cases (5/44 and 8/35) bone resorption was noted.

In a proportion of cases (8/20 and 4/16, respectively) normal spleen cells and bone marrow cells, grafted into pinnae of syngenic BALB/c host animals, also activated perichondral chondrogenesis (Table 4).

Discussion

In the course of localized GvH reaction by injection of parental immunocompetent cells (lymph node, bone marrow, spleen) into F₁ hybrids, activation of perichondrial and periosteal membranes was observed. The intensity of chondro/osteogenesis varied, depending on the amount and type of injected cells. With 25×10^6 of bone marrow cells the stimulation was the highest when compared to 50×10^6 of spleen cells and 20×10^6 of lymph node cells (Table 1, 2). In nearly 30% of cases ectopic chondrogenesis was observed. This suggests that the GvH reaction has triggered intense proliferation of the perichondrial membrane at an earlier phase of the immune reaction.

The stimulation of periosteal osteogenesis and perichondral chondrogenesis was also observed following injection of syngeneic lymphoid cells, either activated *in vivo* by ConA or unstimulated (Tables 3, 4).

The presence of bone trabecules observed in the pinna following bone marrow cell grafting is a manifestation of the osteogenic cell presence in the marrow stromal compartment; these are thus of donor origin (Pfeiffer et al., 1948; Friedenstein et al., 1968; Tavassoli et al., 1971).

The weak response of perichondrium in GvH reaction by lymph node cells could be explained by the relative paucity of grafted cells (20×10^6) and consequent reduction of the immune response.

The GvH reaction in thigh muscles evoked by grafting of 1×10^8 parental spleen cells into F₁ hybrids was more often associated with local bone mass gain (in ca 20% of cases) than with bone loss (in ca 5% of cases). Similar changes in shank bones were obtained earlier following local *in vivo* administration of various immunomodulatory agents (Włodarski and Galus, 1992).

In general, the frequency and intensity of periosteal/perichondreal reaction following localized GvH reaction by parental cells administered subcutaneously or intramuscularly into F₁ hybrids and response following injection of syngeneic cells were similar. The morphological tissue analysis did not reveal any substantial pathological difference observed in either quantitative or qualitative terms. In the present experiments only the dry mass of bones was compared with contralateral controls. Whilst evaluation of changes pertaining to cartilage changes was solely qualitative – a widening of the perichondrial membrane at the site of cell grafting was noted, also the presence of newly-formed chondrocytes or of islands of cartilage outside the perichondreal membrane was observed. The range of such changes varied and sometime they were observed only in a few serial sections.

The systemic introduction of allogeneic lymphocytes into immunocompetent hosts leads to the GvH disease, which is associated with lesions of various tissues (Piguet et al., 1987). When lymphocytes are injected subcutaneously or intramuscularly, the GvH reaction is limited to the injection sites, as was proved by the lack of splenomegaly and by enlargement of lymph nodes restricted to those in the draining area. Similar conclusions were reported by others (Korčáková et al. 1974; Kamiński et al., 1980).The activation of regional lymph nodes following local GvH reaction is manifested also by the increase in the number of lymphocytes with large nucleoli – the evidence of stimulation of RNA synthesis (Korčáková and Hašková, 1974).

Upon activation, cells involved in the inflammatory reaction release a number of growth factors affecting the cells at and adjacent to the site of inflammation (Sasaki et al., 1992; Howard et al., 1993). The presence of inflammatory cells appear to be a general feature in bone formation (Frost et al., 1997). Inflammatory cytokines such as IL-1, TNFß have, among others, a pronounced effect on bone cells: they can enhance osteoblast proliferation and induce bone resorption (Frost et al., 1997; Lane and Goldring, 1998). Histological examination revealed that, during chronic inflammatory reaction, periosteal bone formation is often coupled with osteoclastic bone resorption described on radiograph as a "cotton wool appearance" (Fukawa et al., 1985).

GvH reaction is associated with lymphokine activity production by spleen cells (Hirokawa et al., 1989). It seems possible that during localized GvH reaction various cytokines are produced *in situ* and can act, in a paracrine manner, on neighbouring tissues, for example periosteum, perichondrium and epidermis (Włodarski et al., 1999). Such growth factors may affect a variety of cell populations that have counterbalancing activities. For example, they can activate bone formation and bone resorption simultaneously.

However, it is difficult to explain why the best inducers of the changes in chondrogenesis are bone marrow cells, which are the worst inducers of the GvH reaction in comparison with lymph node or spleen cells. One can only speculate that the presence of chondro/osteogenic precursor cells in the marrow accounts for this phenomenon.

Another explanation for chondro- and osteogenetic activation in association with localized inflammatory reaction (following GvH reaction or following grafting of syngeneic lymphoid cells) could be the triggering of cell activation via purinoreceptors. The damaged cells release purines – ligands for purinoreceptors present on various cell types, bone and epidermis including (Schoefl et al., 1992; Bowler et al., 1995, 1999; Dixon et al., 1999). Extracellular nucleotides released by damaged cells or by controlled secretion could be an important factor in the formation of bone and cartilage. When one considers that purinoreceptors are present on chondrocytes, osteoblasts and osteoclasts (Bowler et al., 1995), it seems possible that nucleotides, released locally in the bone microenvi-

ronment in response to trauma, will react with purinoreceptors expressed by both osteoblasts and osteoclasts to influence the remodeling process (Bowler et al., 1998).

The presence of purinoreceptors in keratinocytes (Dixon et al., 1999) could also explain the proliferation of epidermis following local administration of various immunomodulators and the localized GvH reaction in the pinna (Włodarski et al., 1999).

Acknowledgement

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