Platelet Proteins, Including Platelet-derived Growth Factor, Specifically Depress a Subset of the Multiple Components of the Response Elicited by Glutathione in Hydra

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Abstract. Human serum more strongly depressed the feeding response of Hydra (ball formation) elicited by S-methylglutathione than plasma. On the basis of the effect of several proteins released by platelets, at least five apparent components of the response (R1–R5) were suggested. Each of the platelet proteins examined specifically depressed a subset of these components. Among the platelet proteins examined, platelet-derived growth factor (PDGF) specifically depressed the R2 response (the concentration at which the depressing effect was 50% of the maximum \( ED_{50} \) was 0.17 pM), and basic fibroblast growth factor depressed the R3 and R5 responses (\( ED_{50} 0.50 \) aM) and the R2 response (\( ED_{50} 0.55 \) pM).

With respect to the depression of the R2 response by PDGF, addition of an anti-PDGF IgG or chemical reduction of PDGF, both of which prevent PDGF from binding to its cell surface receptor on responsive cells, eliminated the depressing effect of PDGF on the hydra response. The implications of these observations are discussed.

Reduced glutathione elicits a feeding response in a small freshwater coelenterate, Hydra (29, 31). The response is quantitatively measured in duration of tentacle ball formation, a response associated with feeding (18). The response is depressed by lectins such as Ulex europeus I, Ricinus communis I and II (19), and by dopamine and related amines (20). These agents depress the response elicited by S-methylglutathione (GSM) at concentrations <0.2 \( \mu \)M. The response elicited by GSM at higher concentrations (>0.1 \( \mu \)M) are selectively eliminated, after the animals are illuminated by near UV light in the presence of S-(p-azido-phenacyl)glutathione (21). These observations imply that there are multiple components of the response evoked at different concentrations of glutathione.

Although descendants of interstitial cells (nerve cells and nematocytes) may control the feeding response of Hydra (8, 28), the precise location of a receptor cell or glutathione receptor molecules mediating the response remains to be identified. Recently, while attempting to isolate a monoclonal antibody to the glutathione receptors, we found that serum caused a potent depression of the response. Since plasma depressed the response less potently, proteins released from platelets were evaluated to determine whether they were responsible for the depressing activity detected in serum. Here, we report that there are at least five apparent components of the response evoked by different concentrations of glutathione, and that individual platelet proteins, including platelet-derived growth factor (PDGF), specifically depress only certain subsets of these components.

The platelet proteins show many biological activities: an anti-heparin activity (platelet factor 4 [PF4], reference 33), chemotactic activities for fibroblasts (PF4 and \( \beta \)-thromboglobulin [\( \beta \)TG], reference 41). PDGF is a principal serum mitogen for connective tissue cells (2, 12, 23, 35-37), and also a chemoattractant for fibroblasts and smooth muscle cells (17, 42) as well as monocytes and neutrophils (14, 46). Because of these activities, platelet proteins may play important roles in blood coagulation and wound healing (39).

Materials and Methods

PDGF (human) was highly purified as previously described (35, 36). The molar concentrations of PDGF were calculated assuming 30,000 as its molecular weight. \( \beta \)TG and PF4 were purified from supernatant of frozen and thawed human platelets by heparin-Sepharose column chromatography (30). \( \beta \)TG was further purified by gel filtration on a column of TSK-Gel.
G-3000SW (TSK America Inc., North Bend, WA). Their purity was examined by SDS PAGE and by amino acid analysis. They showed a single band on SDS PAGE in the presence or absence of 2-mercaptoethanol. Their amino acid compositions agreed with those expected from the published sequence of PF4 (13, 24) and 13TG (4). A monospecific anti-PDGF IgG was prepared by Na2SO4 precipitation and DEAE-Sephacel chromatography of plasma from a goat immunized with purified PDGF. At a concentration of 20 μg/ml, the IgG completely antagonized the mitogenic activity of 1 ng/ml of purified PDGF on Swiss 3T3 cells. Highly purified preparation of basic fibroblast growth factor (FGF) from bovine pituitary (15) was a kind gift from Dr. A. Baird (The Salk Institute, San Diego, CA). Pooled human serum and plasma were a kind gift of Dr. T. Iwahashi (University Hospital of Kyushu University). Other reagents were all commercial products.

Epidermal growth factor (EGF) mouse submaxillary gland was obtained from Biomedical Technologies (Cambridge, MA), goat anti-mouse-IgM IgG from Cappel Laboratories (Malvern, PA), and protamine sulfate from Nakarai Chemicals (Kyoto, Japan).

**Assay of the Feeding Response of Hydra**

10 animals of *Hydra japonica* were preincubated with a test substance (serum or purified platelet proteins) in 35-mm diameter dish containing 2 ml Pipes buffer (1 mM Pipes, 1 mM CaCl2, pH 6.2). After 5 min of preincubation, a small amount of concentrated GSM was added into the medium to a specified final concentration, and the dish was gently swirled. GSM is as potent a stimulant as reduced glutathione, and more stable than the latter. The tentacles were motionless in the absence of GSM but, upon stimulation, they were shrunk and intertwined, resulting in a ball in the head region of the animal within 1 min (Fig. 1). The ball formation lasted for 10-20 min depending on stimulatory conditions. The response was determined as an average duration of the ball formation at 20°C as described in reference 18.

The depression of the response by a modulator was expressed as 100 - 100 × (response in the presence of a modulator)/(response in the absence of a modulator) %.

**Assay for Fibroblast Cell Growth**

To examine the cell growth-stimulating activity of bovine sera, 10⁵ cells of human fibroblast MRC-5 (American Type Culture Collection #CCL-171, reference 26, obtained from Flow Laboratories, Inc., MacLean, VA) were plated on 35-mm diameter plastic dish and cultured in Dulbecco's modified Eagle's medium (Difco Laboratories, Inc., Detroit, MI) supplemented with 5% bovine serum (fetal serum, lot 3B021, 99956; M. A. Bioproducts, Walkersville, MD; and neonatal serum, lot 60PB, PC27; Mitsubishi-Kasei Co., Tokyo, Japan) in humidified 5% CO2. The medium was changed and the cell number was determined on days 3, 5, and 7.

**Radioreceptor Assay of PDGF**

The radioreceptor assay for PDGF was performed as described previously (6).

**Results**

**A Depressing Effect of Serum on the Feeding Response of Hydra and Its Potency in Stimulating Cell Growth**

Serum potently depressed the tentacle ball formation of *Hydra* elicited by GSM. The depressing activities of various lots of bovine serum are compared with their growth-promoting activity for human fibroblasts (MRC-5) in Table I. A correlation was noticeable between both the activities.

GSM elicited the feeding response at concentrations >10 nM, but at >100 μM, the response decreased. Pooled human serum (1%) greatly reduced the response in the whole range of GSM concentrations (Fig. 2). Though plasma also reduced the response to GSM of concentrations <100 μM and >20 μM, serum depressed the response in the whole stimulant concentrations at least 10 times more potently than plasma. For example, when the response was examined at 0.2 μM GSM, serum induced 50% depression at a concentration 20-fold lower than plasma (Fig. 3). Along with the correlation between the depression of the hydra response and the growth-promoting activity of various serum (Table I), the difference between plasma and serum (3, 27, 38) suggested the possibility that PDGF may be responsible for the de-

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Figure 1. The feeding response of *Hydra japonica*. (a) The resting animal; the animal is relatively motionless and tentacles are stretched. (b) The animal stimulated with GSM; tentacles are folded and form a ball at the head region (tentacle ball formation). Bar, 2 mm.
Depressing activity of human serum on the feeding response of *Hydra*.

### The Depressing Effect of Alpha Granule Proteins

We examined the depressing effect of a supernatant of frozen and thawed human platelets fractionated on a heparin–Sepharose column, and found that a major active principle was eluted at the same position where PDGF activity was eluted (data not shown). We next examined purified PDGF, which is a major growth factor in serum released from alpha granules of platelets (reviewed in references 37, 39, and 45). Total depression of the response was not achieved with purified PDGF (Fig. 3). The concentration at which the depressing effect was 50% of the maximum (ED$_{50}$) was 0.17 pM for PDGF, which is ~100 times lower than the 33 pM required for half-maximal stimulation of $[^{3}H]$thymidine incorporation into Swiss 3T3 cells (for example, 36). This ED$_{50}$ value is also far lower than the concentrations when PDGF acts as a chemoattractant (14, 17, 42, 46).

We examined the depressing effects of other proteins released from platelets on the response elicited by 0.1 μM GSM (15). The value is ~10-fold smaller than the amount of αTG (170 nM) greatly reduced the response to GSM concentrations >0.1 μM (Fig. 5 B). Near saturating amount of EGF (83 nM) depressed the response to a degree similar to that of the higher dose of basic FGF (Fig. 5 C). A saturating amount of PF4 (170 nM) depressed the response to GSM concentrations <0.1 μM and >20 μM, and decreased the response to GSM concentrations between 0.2 and 10 μM by ~30% (Fig. 5 D). Near saturating amount of βTG (170 nM) greatly reduced the response to GSM at all concentrations examined (Fig. 5 E).

### The Effect of Glutathione Concentration on the Depressing Activity of Platelet Proteins

Since the depressing activity of serum varies with the concentration of glutathione (Fig. 2), we examined the response at different concentrations of stimulant in the presence of near saturating doses of each of the platelet proteins to examine their possible differential effects on the response (Fig. 5). In the presence of 3.3 pM of PDGF, only the response to GSM at concentrations from 0.1 to 1 μM was depressed, and the response below 0.05 μM or above 1 μM was not affected at all (Fig. 5 A). The same results were obtained in the presence of a 10-fold higher concentration of PDGF (data not shown). This observation suggests that PDGF specifically depresses a component of the response at concentrations from 0.1 to 1 μM.

Saturating amount of basic FGF (2.6 pM) depressed the response to GSM concentrations >0.1 μM (Fig. 5 B). The lower dose of basic FGF (0.26 fM) depressed the response to GSM above 1 μM to the same degree as the higher dose, whereas the response below 0.5 μM became near normal (Fig. 5 B). The ED$_{50}$ of basic FGF for the response at 2 μM GSM is 0.50 nM (data not shown), 10$^{6}$-fold lower concentration than required at 0.1 μM GSM (Fig. 4).

At near saturating amount of EGF (83 nM) depressed the response to a degree similar to that of the higher dose of basic FGF (Fig. 5 C). A saturating amount of PF4 (170 nM) depressed the response to GSM concentrations <0.1 μM and >20 μM, and decreased the response to GSM concentrations between 0.2 and 10 μM by ~30% (Fig. 5 D). Near saturating amount of βTG (170 nM) greatly reduced the response to GSM at all concentrations examined (Fig. 5 E).
The depressing activity on the response of platelet alpha granule proteins and protamine. The response of Hydra was determined in the presence of different concentrations of basic FGF (C), EGF (D), purified human platelet βTG (O) and PF4 (⧫), and an arginine-rich basic protein, protamine (●). The animals were stimulated with 0.1 μM GSM. Each point is an average of three to four determinations.

The effect observed at GSM concentrations ~10 μM was resistant to depression, as was observed for EGF and FGF.

The Effect of Modulators of PDGF Binding on the Depressing Activity

An anti-PDGF IgG, which inhibited PDGF binding to its cell surface receptor (Raines, E. W., and R. Ross, unpublished observations), eliminated the depression of PDGF but had no effect on the depressing activity of basic FGF, EGF, βTG, and PF4 (Table II). The anti-PDGF IgG alone at the same concentration did not affect the response to any extent. In contrast, a goat IgG prepared from anti-mouse-IgM serum did not eliminate the depression of PDGF at the same concentration, indicating a specific action of the anti-PDGF IgG on the depressing activity of PDGF.

Two other modulators for the interaction of PDGF with its receptor were also examined. Chemically reduced PDGF is unable to bind to its receptor (46) and is no longer mitogenic. Reduction of PDGF with mercaptoethanol abolished the ability of PDGF to depress the feeding response of Hydra (Table III). Mercaptoethanol alone did not affect the response. Protamine is an arginine-rich basic protein which is able to completely inhibit binding of 125I-PDGF to its cell surface receptor at concentrations of 5 μM (25). This protein is also able to completely inhibit the response at a GSM concentration of 0.1 μM with an ED50 of 0.25 μM (Fig. 4).

The Depressing Activity of Animal Sera

In the above studies, PDGF was the most potent platelet protein acting on the response at 0.2 μM GSM (Figs. 3 and 4). It therefore appeared that the depressing activity at 0.2 μM GSM could be used to estimate the PDGF content in biological fluids. This is also suggested by the effect of anti-PDGF IgG on the depressing activity of animal sera. The anti-PDGF IgG eliminated the major part of the depressing effect of 0.1% human serum, and had a smaller effect on 1% serum (Table II). The PDGF content of various animal sera, both by the depressing on the feeding response of Hydra and by the PDGF radioreceptor assay, are compared in Table IV.

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Figure 5. The effect of various platelet proteins on the response of Hydra. The response was determined at different concentrations of GSM in the absence of any additives (C) and in the presence of (A) 3.3 pM of purified PDGF (●); (B) basic FGF (⧫, 2.6 pM; ●, 0.26 fM); (C) 83 nM of EGF (●); (D) 170 nM of PF4 (⧫); and (E) 170 nM of βTG (●). The response was determined as an average duration (min) of the ball formation. Each point is an average of three determinations. Bar is the standard deviation.
Table II. The Effect of Anti-PDGF IgG on the Depressing Activity of Alpha Granule Proteins and Biological Fluids

<table>
<thead>
<tr>
<th>Additives</th>
<th>Test</th>
<th>Plus anti-PDGF IgG</th>
<th>Plus control IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.0 (reference)</td>
<td>0.3 ± 6.3 (4)</td>
<td>ND</td>
</tr>
<tr>
<td>PDGF 33 pM</td>
<td>59.8 ± 6.5 (4)*</td>
<td>0.0 ± 1.5 (4)*</td>
<td>64.9 ± 1.7 (5)</td>
</tr>
<tr>
<td>Basic FGF 2.6 pM</td>
<td>69.6 ± 1.8 (6)</td>
<td>73.9 ± 4.9 (5)</td>
<td>ND</td>
</tr>
<tr>
<td>EGF 40 nM</td>
<td>77.6 ± 2.0 (3)</td>
<td>75.4 ± 11.8 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>PF4 84 nM</td>
<td>45.1 ± 3.2 (3)</td>
<td>47.7 ± 0.7 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>βTG 39 nM</td>
<td>50.3 ± 1.3 (3)</td>
<td>57.9 ± 6.7 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>Human serum 0.1%</td>
<td>52.0 ± 3.3 (4)*</td>
<td>5.2 ± 4.3 (4)*</td>
<td>54.6 ± 6.4 (4)</td>
</tr>
<tr>
<td>1%</td>
<td>83.4 ± 0.8 (4)*</td>
<td>32.3 ± 5.9 (4)*</td>
<td>ND</td>
</tr>
</tbody>
</table>

* The response was elicited by 0.1 μM GSM in the presence of alpha granule proteins and by 0.2 μM GSM in the presence of human serum. The figures are mean ± SD. The figure in parenthesis is the number of determinations.

The similar approximation by the two different assays, except for fetal bovine serum (see Discussion), suggests that the major depressing activity in animal sera detected in the hydra assay at 0.2 μM GSM may be due to PDGF.

Discussion

Multiple Components of the Response Elicited by Glutathione

From the present observations, at least five components of the response (R1–R5) (Fig. 6) are suggested. Each of them would be defined by a narrow range of stimulant concentrations: R1 at concentrations <0.1 μM, R2 from 0.1 to 1 μM, R3 from 0.5 to 5 μM, R4 from 2 to 20 μM, and R5 at concentrations >20 μM. The response at a specified concentration of GSM may be an integration of these components. The multiple components were also suggested by factors observed previously, such as a differential effect of dopamine on the response to GSM of different concentrations (20), and selective reduction of the response to GSM above 0.1 μM by in vivo photoaffinity labeling (21). Monoclonal antibodies that depressed only one of these components were also isolated (22).

In peripheral sensory systems, multiple receptor units, each of which is specialized in order to respond to a narrow range of stimuli covered by the whole sensory organ, have been observed. This is referred to as range fractionation (10). Glutathione response of Hydra might be a first example of range fractionation with respect to concentration of chemical stimulus. Further studies are required to clarify these multiple components of the response in cellular and molecular organizations, and their biological significance.

PDGF, FGF, and Other Platelet Proteins May Explain the Depressing Activity of Whole Serum

The depressing effects of platelet proteins are remarkable, especially considering the relatively weak effect of plasma proteins (Figs. 2 and 3). Bovine serum albumin, γ-globulins from cow, sheep, goat, and mouse, and bovine insulin showed very little or no effect at comparable concentrations (data not shown).

PDGF and basic FGF appear to be major components of the depressing activities of whole serum. Together with the effect of the anti-PDGF IgG on the depression of whole serum (Table II), the reasonable agreement of PDGF levels estimated by the depressing activities with those by the radio receptor assay (Table IV) indicates that PDGF is the major

Table III. The Effect of Chemical Reduction of PDGF on the Depressing Activity of the Feeding Response of Hydra

<table>
<thead>
<tr>
<th>Additives</th>
<th>Depression*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Intact PDGF 0.33 pM</td>
<td>48.3 ± 6.5 (4)*</td>
</tr>
<tr>
<td>Reduced PDGF§ 0.33 pM</td>
<td>3.2 ± 3.4 (3)*</td>
</tr>
<tr>
<td>Mercaptoethanol alone</td>
<td>1.3 ± 2.1 (3)</td>
</tr>
</tbody>
</table>

* The response was elicited by 0.1 μM GSM.
† Significantly different (P < 0.01) by Student's t test.
§ One part of purified PDGF solution (1 μg/ml) was mixed with nine parts of 10 μM mercaptoethanol, 50 mM Tris, pH 7.5. After a brief incubation at room temperature, the mixture was diluted to 10-fold with Pipes buffer for the behavioral assay. Because of a harmful effect of mercaptoethanol on the intact animals, a high dilution of mercaptoethanol, and thus a low concentration of PDGF, was used.
†† The effect of mercaptoethanol alone at the same concentration used to reduce PDGF (see footnote §).

Table IV. The PDGF Levels in Animal Sera Estimated by the Depressing Activity and by PDGF Radioreceptor Assay

<table>
<thead>
<tr>
<th>Animal serum</th>
<th>PDGF estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By depression*</td>
</tr>
<tr>
<td></td>
<td>ng/ml</td>
</tr>
<tr>
<td>Fetal bovine</td>
<td>204 ± 44</td>
</tr>
<tr>
<td>Human</td>
<td>22.3 ± 9.4</td>
</tr>
<tr>
<td>Bovine</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Mouse</td>
<td>10.0 ± 3.8</td>
</tr>
<tr>
<td>Horse</td>
<td>0.40 ± 0.1</td>
</tr>
</tbody>
</table>

* The depression against the response elicited by 0.2 μM GSM was determined in the presence of three different doses of the relevant sample. The PDGF levels were determined from these depression values, as indicated in Fig. 3. Values are mean ± SD.
† The PDGF levels were determined by radioreceptor assay for the same samples as used for the depression assay.
active principle in whole serum responsible for the depression of the R2 response. Basic FGF depressed the R3 and R5 105-fold more potently than it did the R2 response (Fig. 5 B). It appeared to be similarly potent for both R3 and R5 (data not shown). As the R3 response was depressed by basic FGF more specifically than the R5 response (Fig. 5 B), FGF activity may be estimated by the depression of the R3 response with less interference. EGF and βTG depress the R3 response only at high concentrations (Fig. 5, C and F, data not shown). We could estimate the basic FGF content to be 0.76 pg/ml by analyzing the depression of pooled human serum on the R3 response (data not shown).

The major component that depresses the R1 response is not clear at present. βTG and PF4 might be candidates, but an unknown component is likely, considering their levels in serum (6) and the relatively high concentrations required to depress the response.

Fetal bovine serum contained an activity potent enough to depress the R2 response (Tables I and IV). Though PDGF level in fetal bovine serum is low (0.83 ± 0.24 ng/ml, Raines, E. W., and R. Ross, unpublished observation and Table IV), the basic FGF-like activity, which was estimated from the depression on the R3 response, was high enough to explain its depressing activity on the R2 response (data not shown). The principal depressing activity of fetal bovine serum appears to be due to basic FGF or a related substance.

**PDGF Modulation of the Feeding Response Appears to Be Receptor Mediated**

The depressing activity of PDGF on the R2 response was modulated by all the investigated treatments that interfered with the interaction of PDGF with its cell surface receptor. A basic protein, protamine, potently depressed the feeding response at 105-fold higher concentrations than PDGF (Fig. 4, essentially the same depression was observed when the response was elicited by 0.2 μM GSM, data not shown). This is the same concentration difference as seen in competition between 125I-PDGF and protamine for the cell surface receptor on responsive cells. The anti-PDGF IgG specifically eliminated the depressing effect of PDGF on the feeding response and this treatment prevented the binding of PDGF to its cell surface receptor (Raines, E. W., and R. Ross, unpublished observations). Finally, chemical reduction of PDGF, which destroys the ability of PDGF to bind to its cell surface receptor (46), also eliminated the depressing activity of PDGF on the feeding response. Together with the observation that PDGF modulates only a specific component of the response to glutathione, these results suggest that a specific PDGF receptor in Hydra modulates its feeding response. The protozoan Tetrahymena has also been reported to possess a PDGF receptor (1).

**Platelet Protein Homologues in Lower Organisms and Biological Implications**

Hydra has a strong regenerating potential when it is excised at its body column (44). Potent depressing activities were released from excised animals (Hanai, K., unpublished observations). Though further investigations are required, these activities may be due to platelet protein homologues in lower organisms. A phylogenetic analysis of clotted blood serum by radioreceptor assay found a PDGF homologue in the blood of all members of phylum Chordata, but nothing detectable below this phylum (43). However, the depression of the feeding response is more sensitive to the platelet protein homologues and appears to be less specific, even as to the R2 response, than the vertebrate PDGF receptor.

Immunoreactivities to neuropeptides such as FMRFamide, and oxytocin/vassopressin have been detected in hydros tissues (reviewed in 16). The head activator neuropeptide, which was first isolated from hydros tissues (40), was also found in human tissues (5). These observations suggest a primordial significance of peptides as information mediators in lower animals. There would be a close relationship between feeding and growth. Feeding results in cellular proliferation, as indicated by a sharp, phasic increase of mitotic index in several cell types of Hydra (7, 11). That is, it seems likely that this process triggers the release of growth factors in addition to supplying nutrition. It is also possible that growth factors may directly participate in the regulation of food intake. The platelet protein homologues may play more multiple roles than their vertebrate counterparts.

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