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Biochemistry

Adsorptive endocytosis of fibrin monomer by macrophages: Evidence of a receptor for the amino terminus of the fibrin α chain

(heme-octapeptide/cytochemistry/fibrin complexes/Gly-Pro-Arg)

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ABSTRACT **Human fibrinogen and fibrin monomer were labeled with heme-octapeptide for cytochemical examination of their interaction with rabbit peritoneal macrophages *in vitro*. Upon short exposure to labeled fibrin monomer in solution with unlabeled fibrinogen, the cells became covered with surface-adsorbed monomer in nonaggregated form and having a characteristic trinodular shape. Within 30 min, the adsorbed monomer became fully internalized by vesicular uptake, with much of it being incorporated into lysosomal bodies. A concomitant loss of adsorptive capacity of the cell surface for uptake of more monomer accompanied the internalization. By contrast, labeled fibrinogen was not adsorbed by the macrophage surface. Some internalization of fibrinogen by passive fluid-phase pinocytosis was evident, but it was not accompanied by loss of adsorptive capacity for fibrin monomer. The active uptake of monomer may have depended on binding to the amino terminus that is blocked by fibrinopeptide A in fibrinogen, because addition of synthetic peptide corresponding to the terminal Gly-Pro-Arg segment inhibited both the adsorption and the internalization of monomer.**

Molecular forms of fibrin are transported in a soluble state as complexes with fibrinogen in blood (1, 2), and their clearance from the circulation depends largely upon uptake by phagocytic cells (3, 4). The clearance of fibrin from blood has usually been viewed as involving phagocytosis of microclots (3, 5). However, observation of substantial binding of soluble fibrin complexes by macrophages (6) has raised the possibility that the clearance mechanism may not depend on aggregation into an insoluble deposit. There appear to be two distinct modes of uptake of dissolved fibrin: (*i*) a rapid one that is saturable (5) and is seemingly not dependent on plasma fibronectin as a cofactor, and (*ii*) a relatively slow one that is not saturable (7) but is dependent on plasma fibronectin. A third mechanism has been suggested (8), that microclots become degraded by fibrinolytic enzymes and the degraded fibrin is absorbed.

To provide means for assessing the physical state of dissolved fibrin in the course of its absorption, we sought to label it for visualization in the electron microscope. By labeling with heme-octapeptide (9) we were able to detect characteristically trinodular fibrinogen and fibrin cytochemically at the molecular level, and we have detailed a mode of uptake of fibrin in monomeric form that involves adsorptive endocytosis through a monomer-specific receptor, which we infer has high affinity for the fibrin aggregation site that is masked by fibrinopeptide A in fibrinogen.

The labeling was based on ability of the heme-octapeptide to catalyze peroxidatic formation of osmiophilic, electron-dense staining reaction product from diaminobenzidine. Heme-octapeptide had been used, heretofore, mainly by itself as a "microperoxidase" (10, 11) for electron microscopic studies on permeability. Kraehenbuhl *et al.* (9) employed it as a conjugate with Fab fragments of antibody for immunocytochemical staining of IgA secretory organelles. Possibly because of the very high concentrations required for intense staining, the peptide had not been used for direct labeling of macromolecules. However, the prospect of labeling large proteins with several moles of heme-octapeptide to achieve intense staining within the confines of the macromolecule had not been explored.

MATERIALS AND METHODS

Heme-Octapeptide. Heme-octapeptide was prepared and then modified through reaction with a *N*-hydroxysuccinimide ester of carboxybenzaldehyde as described (9) to add an aldehydic function for attachment to protein by reductive amination. The reaction with the ester was repeated two to three times until electrophoresis at pH 2 showed 90% conversion to the carboxybenzaldehyde derivative, which was then separated from excess ester by gel filtration as described (9) and freeze dried.

Fibrinogen was freed of cryoprecipitable monomer (12) and purified further as chromatographic peak 1 (13) free of factor XIII, plasminogen, and plasma fibronectin, and was stored frozen in (9/1) 0.3 M NaCl/0.3 M sodium phosphate (pH 7.4) with Trasylol (100 kallikrein inhibitor units/ml) and 0.1 mM EDTA added as preservatives. The fibrinogen was verified to be free of dissolved fibrin by absence of fast-sedimenting peaks (>8S) or shoulders in the ultracentrifuge, and by absence of a leading shoulder (14) on gel filtration of concentrated solutions (>20 mg/ml) through 4% agarose gel equilibrated with a 1:1 mixture of 0.3 M NaCl and 0.3 M Tris·HCl at 25°C. Just before labeling, it was precipitated and washed with 13% (vol/vol) ethanol at 0°C and redissolved (≈10 mg/ml) in 0.3 M NaCl/phosphate (pH 7.4). The fibrinogen was admixed with specified molar proportions (20% in excess of the desired degree of labeling) of aqueous 4 mM carboxybenzaldehyde derivative of the heme-octapeptide for 1 hr, and then with 1/5 vol of 0.2 M $NaCNBH_3$ (15) in 0.3 M NaCl/phosphate overnight at ambient 25°C. One-tenth volume of 0.3 M Tris·HCl at pH 7.4 was then added to provide a low pK_a amine for displacing unreacted portions of the aldehyde from the fibrinogen. The labeled fibrinogen was separated from unbound heme-octapeptide by gel filtration through 8% agarose in (9/1) 0.3 M NaCl/0.3 M Tris·HCl at pH 7.4. With numerous preparations, chromatograms monitored by absorbance at both 280 and 405 nm showed approximately 80% of the heme-octapeptide co-eluting with fibrinogen (V_e/V_i = 0.4, in which V_e is elution volume and V_i is included volume) and unbound heme-octapeptide well separated near the end (V_e/V_i = 1). The

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heme-octapeptide associated with fibrinogen after gel filtration chromatography was covalently bound, as evidenced by non-removal with sodium dodecyl sulfate, urea, or trichloroacetic acid. The heme-octapeptide to fibrinogen ratios were determinable from absorbances (A) and molar extinction coefficients (ε) of the protein (F) and the heme-octapeptide (H) at 280 and 405 nm, 405 nm being an isosbestic point in the spectra from unreacted and reacted heme.

$$[H]/[F] = \frac{A_{405}/\varepsilon^{H}_{405}}{[A_{280} - (A_{405} \times \varepsilon^{H}_{280}/\varepsilon^{H}_{405})]/\varepsilon^{F}_{280}}$$

$$= \frac{A_{405}/5.6 \times 10^4}{(A_{280} - 0.332A_{405})/5.1 \times 10^5}.$$

Fibrin Monomer. Unlabeled fibrin monomer was prepared by treating fibrinogen with thrombin at pH 5.3 as described (12) for preferential removal of fibrinopeptide A. Labeled fibrin monomer was prepared from labeled fibrinogen by essentially the same procedure, except that pH 5.6 was used because of low solubility of the heme-octapeptide-fibrinogen at pH 5.3. After tests (12) showed 95% conversion to monomer, the thrombin was inactivated with hirudin in 10-fold molar excess of the thrombin.

Solutions of fibrinogen and fibrin monomer used for cytochemistry were prepared by diluting the concentrated proteins (>1:20) with Hanks' balanced salt solution containing predetermined amounts of added Tris to adjust to pH 7.4 and also containing rabbit serum albumin (10 mg/ml) as surfactant. The final solution contained fibrinogen at 1 mg/ml and monomeric fibrin at 0.04 mg/ml, the large excess of fibrinogen being required to maintain the fibrin in a fully soluble state (1, 16).

Macrophages. The macrophages were derived from rabbits by peritoneal lavage 3 days after injection of mineral oil as in studies of Sherman and Lee (6). The cell suspension, collected in phosphate/saline with 10 units of heparin (17), was washed twice by centrifugation in Hanks' solution containing heparin and was resuspended with 20% fetal calf serum added. Cells ($2–5 \times 10^6$) were added to 35-mm plastic culture dishes and after a 2- to 3-hr incubation the nonadherent cells were removed with two vigorous washes. The adherent cells were cultured at 37°C in 95% air/5% CO_2 for 2–3 hr. For transmission electron microscope examination of whole cells, the macrophages were grown for 24 hr on plastic coverslips containing carbon-coated Formvar gold grids.

Cytochemistry. Cytochemical studies were carried out by first rinsing the cells with Hanks' solution containing albumin at 10 mg/ml and then adding 3 ml of labeled protein in albumin solution for allotted periods as specified at 37°C. The cells were then rinsed three times with albumin solution and fixed with 2% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate containing 4.4% sucrose at pH 7.2 for 15 min. They were then washed three times with cacodylate/sucrose and exposed to 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) and H_2O_2 (0.02%) in 0.05 M Tris·HCl according to the method of Karnovsky (18) as modified by Steinman and Cohn (19). The stained cells were washed again with cacodylate/sucrose and then postfixed for 15 min with 1.0% OsO_4 in cacodylate/sucrose at 25°C. For *en face* sectioning, the postfixed cells were dehydrated gradually with alcohol and embedded in Epon 812. For stereoscopic examination of whole cells, the grids with attached cells were dried at the critical point.

Fibrin clots were prepared in thin films according to Bang *et al.* (20) for microscopy on Formvar-coated gold grids but were stained only by the diaminobenzidine and osmium procedures used for cytochemistry. To minimize surface adsorption of the fibrinogen, the Formvar supports were rinsed with albumin solution before the fibrinogen solution employed for clot formation was added.

RESULTS

Properties of the Conjugates. Fibrinogen that was labeled with 10 hemes per molecule formed clots with a slightly faster onset (63 sec vs. 75 sec for control) as detected from turbidity change measured at 315 nm and from gelation on exposure to thrombin. Increasing the degree of labeling to 19.5 hemes per molecule caused a retardation of clot formation (240-sec vs. 75-sec onset, and 10 min vs. 8 min for 80% completion). Clot structure with 10 hemes per molecule of fibrin appeared normal as assessed initially from the turbidity change and verified by electron microscopy. There was an acidic shift in isoelectric point, which caused the solubility of monomer preparations to become less than normal at pH 5.3, whereas the solubility at the normal pI of 5.6 became enhanced. Electrophoretic analysis (4) of fibrinopeptides released by thrombin showed normal yields within 10% of theoretical, an indication that very little of the heme-octapeptide had become linked to the amino terminus; presumably it had combined with the abundant ε-amino groups. Release of fibrinopeptide A at pH 5.3 or 5.6 for production of fibrin monomers with fibrinopeptide B intact proceeded without aggregation as anticipated (21), and the resultant monomers coagulated fully when admixed with buffer at pH 7.4 and remained soluble (between 0.1 and 0.2 mg/ml) when admixed with neutral fibrinogen (1 mg/ml) for formation of soluble complexes (1, 16).

Peroxidase activity of the heme-octapeptide itself was reversibly (22) inactivated by cyanide (23) and by cyanoborohydride. When the heme-octapeptide was separated from excess cyanide and cyanoborohydride by gel filtration through Bio-Gel P-2 (Bio-Rad) its peroxidatic activity returned to normal. The peroxidatic activity (24) of the fibrinogen-linked peptide towards *o*-dianisidine at pH 7.2 was only 10% of that of the nonconjugated peptide, a reduction comparable to that observed in analogous studies by Kraehenbuhl *et al.* (9). The reaction product from peroxidation of diaminobenzidine with heme-labeled fibrinogen caused the fibrinogen to precipitate from solution, but when the product was produced in mixtures of labeled and unlabeled fibrinogen (0.02 mg labeled and 0.7 mg unlabeled per ml), only a slight (7%) precipitation of the unlabeled fibrinogen ensued while the labeled protein precipitated fully (the measurements being made with radioiodinated proteins). This observation provided an indication that the reaction product was binding predominantly to the labeled molecule producing it and was not diffusing substantially to the unlabeled

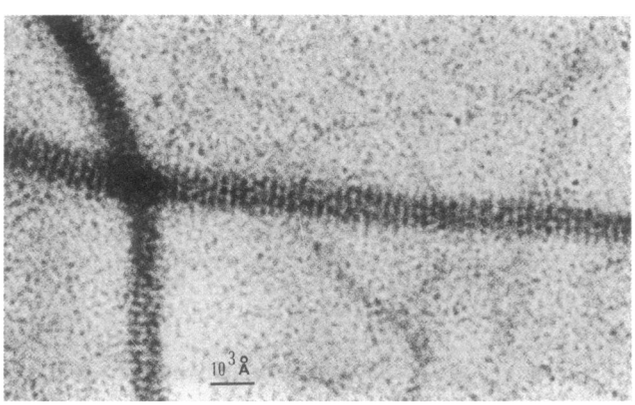

FIG. 1. Fibrin strands formed from heme-labeled fibrinogen and stained by the cytochemical procedure.

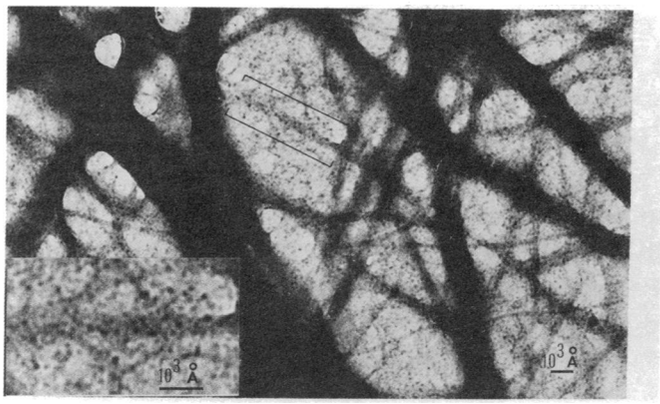

FIG. 2. Fibrin strands formed from a 20:1 mixture of unlabeled and labeled fibrinogen exhibited densely staining trinodular bodies resembling fibrin monomer. The area in brackets is magnified in the *Inset*.

molecules. Further evidence for this was obtained from electron microscopy.

When the labeled fibrinogen was coagulated with thrombin and subsequently subjected to the cytochemical procedure, the electron-dense reaction product was seen to be deposited in the form of nodular bodies arrayed with a periodicity of 238 ± 20 Å within the fibrin strands (Fig. 1). To determine whether the dense staining bodies corresponded to the nodular domains (25–28) of fibrin molecules within the strands, clots from mixtures of labeled and unlabeled fibrinogen were examined. With a large (20:1) excess of unlabeled fibrinogen, the dense staining was still observed to be nodular, but it was sparsely distributed without periodicity within the strands, and triads resembling the trinodular shape of the protein became evident on examination of thin strands (Fig. 2). The length of the triads (485–525 Å) was near that (450–490 Å) characteristic of fibrinogen, but thickness of the nodules (120 ± 40 Å) was somewhat greater than that (70–90 Å) observed with shadowed (26) and with unstained (27) fibrinogen, the added thickness conceivably arising from the reaction product.

Cellular Uptake. Macrophages were exposed to dilute (0.04 and 0.01 mg/ml) labeled monomer (12 hemes per molecule) in solution with unlabeled fibrinogen (1 mg/ml) and processed as described for cytochemical localization of the peroxidase label. Qualitatively similar results were obtained with both levels of monomer employed. Within 4 min of exposure to the labeled protein, the cells exhibited a thin (90- to 120-Å) rim of densely staining reaction product from monomer adsorbed to the cell surface as seen in sections of ≈800 Å thickness (Fig. 3). Further, the dense staining appeared nodular. When viewed by transmission of the electron beam directly through the whole cells (Fig. 4), the densely staining bodies resembled the applecore-like structure of the monomers as seen in other modes of microscopy (25, 26). Further, the monomers appeared discrete with little or no clustering, and fibrin strands were absent.

After 30-min incubation with the cells, adsorbed monomers became almost entirely cleared from the surface of the cells due to internalization into vesicles and vacuoles, many of which were associated with lysosomal bodies (Fig. 3). The absence of all but occasional particles of monomer associated with cell surface provided an indication that a loss of adsorptive receptors for the monomers accompanied the internalization, and this was verified by the absence of new uptake of monomer after the cells had been washed and reexposed to monomer/fibrinogen solution for 4 min.

Cells that were exposed to labeled fibrinogen (0.2 mg/ml) exhibited no dense staining at the cell surface after 4- or 30-min incubation (Fig. 5), an indication that surface binding of the labeled monomer could not be attributed to an affinity for the heme-octapeptide label. Some internalization of labeled fibrinogen into vesicles and vacuoles was observed after 30 min, presumably due to fluid-phase pinocytosis, no adsorption being observed. The densely staining particles were not tightly packed in the vesicles as were the monomers that were internalized after adsorption to the surface. However, much of the internalized fibrinogen appeared somewhat clustered, possibly due to precipitation by the glutaraldehyde fixative.

Because fibrin monomer differed chemically from fibrinogen only in lacking fibrinopeptide A, and because the monomers were adsorbed in nonaggregated form, the possibility could be considered that the adsorption occurred through an epitope that is masked by fibrinopeptide A in fibrinogen. The tripeptide Gly-Pro-Arg, which corresponds to the first three amino acids located behind fibrinopeptide A in fibrinogen (29) and which has been implicated (30) as the epitope through which fibrin undergoes aggregation, was accordingly examined for possible effect upon uptake of the monomers by the cells.

When synthetic (31) Gly-Pro-Arg was added at 0.1 mM (250 equivalents in excess of the fibrin, 10-fold in excess of fibrinogen, and equimolar with albumin), uptake of the monomer became greatly retarded. Virtually no surface adsorption was detected within the 4-min exposure that had yielded a densely staining rim of surface-adsorbed monomer in the absence of the tripeptide. After 30-min exposure with the peptide added, adsorption did reach a level resembling that observed at 4 min in absence of the tripeptide; however, very little of the monomer

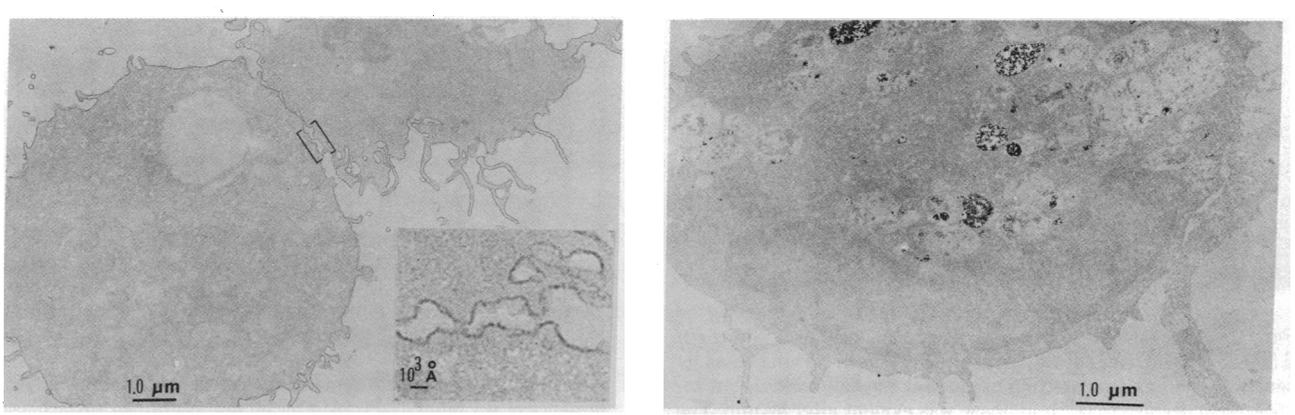

FIG. 3. Sections of macrophages showing rim of cytochemical reaction product from heme-labeled monomer that became adsorbed within 4 min (*Left*), and full internalization of the labeled monomer within 30 min (*Right*). The area in brackets is magnified in the *Inset*.

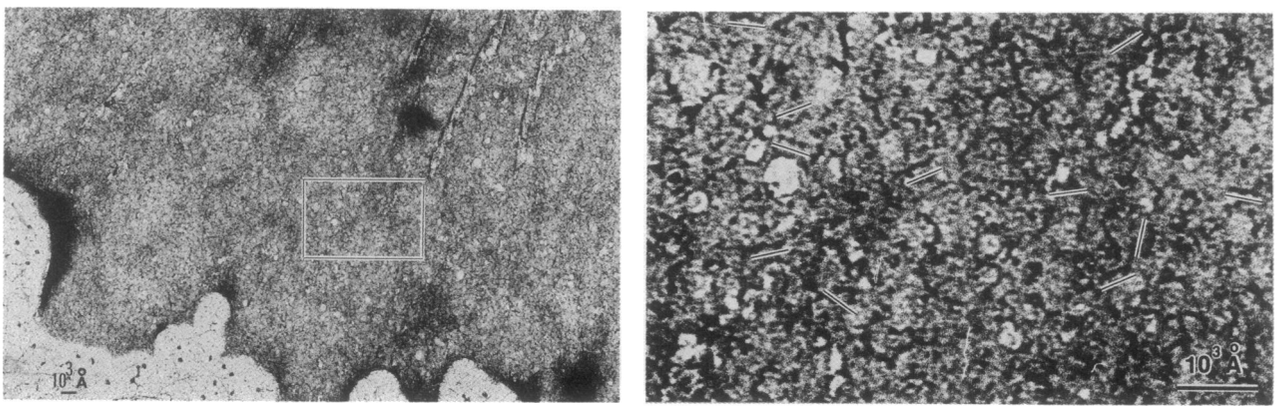

FIG. 4. *En face* view of spread cell 4 min after exposure to heme-labeled monomer, exhibiting densely staining bodies resembling discrete molecules. (*Right*) Enlargement of the area outlined in *Left*. Bars emphasize trinodular structures.

became internalized, and the occasional vesicles found to contain the probe were not of the densely filled type formed in the absence of the tripeptide (Fig. 6).

DISCUSSION

The results of this study warrant the inference that a receptor for uptake of fibrin monomer by macrophages exists and has high affinity for the amino-terminal region of the fibrin α chain. Rapid adsorption of nonaggregated monomer lacking fibrinopeptide A, the nonadsorption of fibrinogen with fibrinopeptide A intact, and the inhibition of uptake by synthetic Gly-Pro-Arg provide the principal justification for this inference.

The Gly-Pro-Arg segment at the amino terminus of the fibrin α chain has also been implicated as an aggregation site (30). If cellular uptake and aggregation of fibrin monomer depend on the same epitope, uptake could depend on access of the cells to the fibrin in nonaggregated or dissociated form because the epitope may not be accessible while occupied in aggregation. Likewise (32), clearance of fibrin complexes from blood may depend on their dissociability. Differences in detail between our *in vitro* observations and those of Sherman and Lee (6) conform with this corollary of an interdependence between dissociation and uptake. Surface uptake of monomer in the present study appeared to reach saturation within a time on the order of 4 min, whereas saturation was not reached until approximately 40 min in their study. The monomer employed in their studies lacked fibrinopeptide B in addition to A, and such monomers have been shown to form complexes with fibrinogen that are relatively nondissociable (33, 34) compared to the readily dissociable type (35, 36) formed by monomers lacking A alone, as employed in the present study. Further, relatively large quantitites of complexes on the order of 10 times the monomer employed here were required for saturation of adsorptive capacity of the cells, another indication that efficiency of uptake may have been impaired by low dissociability.

Saturative binding in the present study can be inferred from loss of adsorptive capacity after internalization of initially bound fibrin. Some desorption could conceivably have occurred through proteolysis at the cell surface, but even freshly added monomer failed to become adsorbed. This loss of adsorptive capacity warrants speculation that depletion of receptors contributes to the blockade of fibrin clearance implicated as a course of fibrin deposition in the generalized Shwartzman reaction (3).

Jilek and Hörmann observed that trypsinized macrophages absorbed fibrin complexes over a 24-hr period by a nonsaturable mechanism dependent on plasma fibronectin. Because plasma fibronectin has been shown to facilitate absorption (37) of mac-

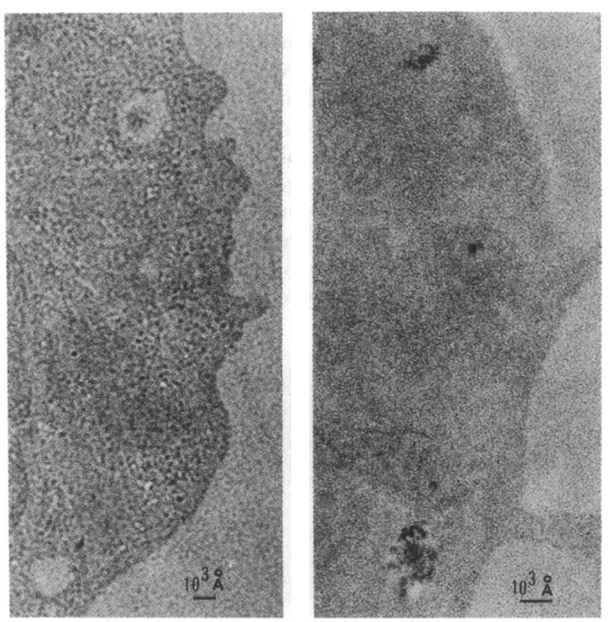

FIG. 5. Cells exposed to heme-labeled fibrinogen for 4 min (*Left*) and 30 min (*Right*). Nonadsorption of the fibrinogen was indicated by absence of cytochemical reaction product at the cell surface, but internalization deemed to be fluid phase was evident after 30 min. The concentration of heme-labeled fibrinogen (0.2 mg/ml) was 5 times that of heme-labeled monomer employed in the experiments of Figs. 3 and 4.

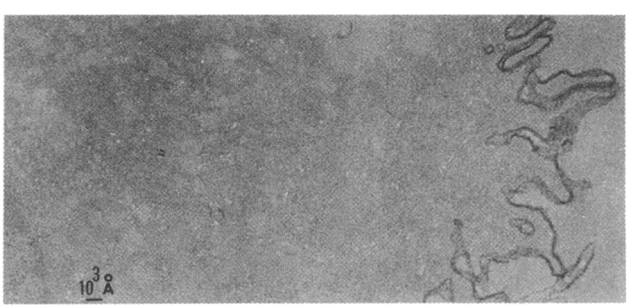

FIG. 6. Cell exposed to heme-labeled monomer (0.04 mg/ml) in the presence of 0.1 mM Gly-Pro-Arg for 30 min contained surface-associated monomer (not seen at 4 min) and differed from controls that internalized virtually all adsorbed monomer in the absence of the tripeptide.

roaggregated albumin (38, 39), an analogous effect on absorption of fibrin complexes might depend on their aggregation, a condition just the converse of the dissociation that we imply to be essential to the receptor-mediated uptake. Plasma fibronectin was absent from the fibrin and fibrinogen preparations employed in the present study, but it was considered by Sherman and Lee (6) to have no effect on saturable uptake.

The binding of fibrin monomer to the macrophage surface may be viewed as resembling its binding to fibrinogen with regard to the inhibition by Gly-Pro-Arg (30). Studies by Colvin and Dvorak (17) have raised the possibility that a calcium-dependent fibrinogen receptor exists on the macrophage surface. If so, the binding and the internalization of fibrin monomer could conceivably involve some cooperative interaction with adsorbed fibrinogen. Although we observed no adsorption of added fibrinogen under the conditions of the present study, our studies were patterned after those of Sherman and Lee (6), who also obtained a negative result. Many plausible explanations remain to be studied for clarification of the adsorption mechanism.

Apart from the details provided for the saturable uptake of monomer, the results of the present study demonstrate the utility of heme-peptide labeling for preparation of cytochemical probes. Because of the relatively low peroxidase activity of the heme-peptide (9) compared to other peroxidases, particularly horseradish peroxidase (18, 24), used for cytochemistry we were concerned whether adequate staining intensity could be achieved to enable us to observe the labeled protein. However, the small size of the heme-peptide enabled us to attach a large number without seriously altering properties of the fibrinogen, while imparting a high concentration of the peptide within the confines of the fibrinogen molecule. Dense staining obtained within the confined space yielded images resembling the known trinodular shape of fibrinogen. It is anticipated that similar labeling can be achieved with other proteins, at least for resolution within 60 to 90 Å, the size of the nodular domains resolved in the present study.

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