The Pathogenesis of coronary and cerebrovascular thrombosis has been traditionally interpreted as a "misdirected" form of hemostasis in which platelets interact with subendothelium, leading to adhesion, activation, aggregation, and recruitment, accompanied by fibrin formation. This concept evolved mainly from studies of the response of healthy blood vessels to physical injury and formed the basis for current platelet-directed therapeutic approaches to arterial thrombosis. However, thrombotic events almost always occur at sites of pathologic vascular damage, containing lipid-rich or necrotic tissue and inflammatory cells. Dis- turbance of blood flow at these sites promotes interactions between multiple cell types.

Time course studies of the morphology of an evolving thrombus always show participation of several cell types, in addition to the normal endothelial cells surrounding the lesion. Platelets form the central core of the thrombus, which then becomes admixed with erythrocytes, neutrophils, and later monocytes. Physical participation of multiple cells was initially thought to be passive. However, our in vitro studies involving reactions of endothelial cells, erythrocytes, and neutrophils with platelets led us to consider thrombosis as an integrated group of multicellular events. Cell contact and varying degrees of pathologic stimulation govern development and reversibility of thrombosis. We hypothesize that thrombotic and inflammatory responses are biochemically linked as part of overall mechanisms of host defense. The physical and biologic behavior of one cell can be markedly altered by the presence of another. This result can be due to direct cell contact or to secretory products of one or more cell types, especially after activation by specific agonist(s). Eicosanoids. In 1979 we devised an in vitro model system to investigate platelet-endothelial cell interactions under conditions of motion and cell contact. This investigation was performed with combined suspensions in aggregometer cuvettes to achieve maximal cell density in a small volume. These parameters were not achievable in experiments with endothelial cell monolayers. Furthermore, we were thereby able to study function and biochemistry simultaneously in an in vitro model of thrombosis.

The presence of endothelial cells in suspension invariably led to instantaneous loss of platelet reactivity, irrespective of the platelet agonist used. We attributed this consistent inhibition of platelet function to synthesis and release of prostacyclin (PGI,) from endothelial cells. Our experiments demonstrated that activated platelets participated in endothelial cell synthesis of PGI via release of endoperoxide and free arachidonate--precursors for production of PGI,. This phenomenon is now known as transcellular metabolism. In these reactions one cell uses precursors and intermediates from another for production of a new metabolite(s) with entirely different functions. These new metabolites include compounds that cannot be formed by either cell alone. Thus, transcellular biosynthesis of eicosanoids via reactive precursors and intermediates is a critical form of cell-cell communication. Occurrence of this phenomenon has recently been shown in erythrocytes, neutrophils, and smooth muscle cells, in addition to platelets and endothelial cells. Trans-

PLATELET-ENDOTHELIAL CELL INTERACTIONS

Currently, there is evidence for at least three endothelial cell-derived thromboregulators: eicosanoids, endothelium-derived relaxing factor(s), and ecto-nucleotidase(s) (Table 1).
cellular metabolism may also explain previously unanswered clinical questions in thrombotic diseases.\textsuperscript{13} For example, an aspirin-treated platelet can regain its capacity as a vasoconstrictor by metabolizing released neutrophil LTA\textsubscript{4} to LTC\textsubscript{4}.\textsuperscript{11} The latter can replace the biologic effects of thromboxane on blood vessels—a property abolished by aspirin.\textsuperscript{13}

Inhibition of platelet reactivity via blockade of cyclooxygenase constitutes the theoretical basis of clinical trials with aspirin. The successful outcome of these trials has been mainly attributed to elimination of TXA\textsubscript{2} production and its consequent inhibition of platelet reactivity.\textsuperscript{14,16} It may eventually be found that cyclooxygenase inhibition exists in conjunction or synergy with additional biochemical attributes of aspirin, contributing to the total therapeutic effectiveness of this drug in vascular disease. Clinical use of aspirin as an antithrombotic agent was originally based on its properties as an antithrombotic modality, as evidenced by prolongation of the bleeding time. This phenomenon was subsequently found to be a direct consequence of cyclooxygenase inhibition. Whether it is scientifically logical to extrapolate consequences of injury to a healthy blood vessel (as in this test of hemostasis) to activation of platelets in the microenvironment of a fissured atherosclerotic lesion,\textsuperscript{3} including its necrotic and cellular components, is debatable.

Thus, we hypothesize that the effectiveness of aspirin in thrombosis may be due in part to other biochemical effects in addition to cyclooxygenase inhibition. Modulation by aspirin of the activity of other thromboregulatory mechanisms such as those in Table 1, would conceivably contribute to the total antithrombotic potential of this drug.

We had originally attributed loss of platelet reactivity in the presence of endothelial cells to PG\textsubscript{1}, formation, both endogenously and via transcellular metabolism of precursors from platelets. It is now clear that at least two other substances (endothelium-derived relaxing factor [EDRF] and endothelial membrane ecto-adenosine diphosphatase [ecto-ADPases]) can also regulate platelet reactivity at the vessel surface. Importantly, neither EDRF nor ADPases are affected by aspirin.

EDRF. This fluid-phase autacoid was discovered in endothelium by Furchgott and Zawadzki in 1980.\textsuperscript{11} It has now been found in a variety of tissues,\textsuperscript{8,20} including collagen-stimulated platelets.\textsuperscript{21} In response to chemical or physical perturbation, vascular endothelium releases EDRF, which induces marked vasodilation and inhibition of platelet adhesion and aggregation. Most investigators now believe that the biologic activity of EDRF can be accounted for by nitric oxide (NO).\textsuperscript{22,23} EDRF stimulates the soluble guanylate cyclase in smooth muscle and platelets, elevating intracellular cyclic guanosine monophosphate (cGMP).\textsuperscript{24,25} Recently, L-arginine was identified as precursor of NO.\textsuperscript{27} Most EDRF studies to date on isolated endothelial cells have been performed with animal cells cultured on microcarrier beads. Biologic activity has mainly been evaluated on preconstricted vessel strips, and much less frequently by inhibition of platelet aggregation.

EDRF is difficult to study because of a half-life of approximately 5 seconds. It is inhibited by superoxide radical, hemoglobin, ferrous iron, and methylene blue. The duration of biologic activity of EDRF is extended by superoxide dismutase (SOD) and inhibitors of cGMP phosphodiesterase. EDRF belongs to the group of agents known as "nitrovasodilators." These nitrovasodilators lead to formation of the reactive nitric oxide free radical (-N = O). It has been reported that production of EDRF in atherosclerotic vessels is impaired.\textsuperscript{26-30}

Broekman et al recently demonstrated EDRF activity in combined suspensions of thrombin-stimulated human umbilical vein endothelial cells (HUVEC) and platelets.\textsuperscript{31} Platelet aggregation and serotonin release were inhibited in parallel under these conditions.\textsuperscript{31} Importantly, HUVEC-derived EDRF was transferable in the fluid phase, enhanced by SOD, inhibited by hemoglobin, and aspirin-insensitive. In addition, EDRF activity could be blocked by derivatives of L-arginine with modified guanidino groups, in accordance with the putative pathway of NO formation. There are now suggestions in the literature that impairment in EDRF production plays a critical role in hypertension, atherosclerosis, and vascular occlusion.\textsuperscript{32} EDRF activity may serve as a reinforcing mechanism in thromboregulation when PG\textsubscript{1}\textsubscript{2} production is insufficient, or has been eliminated by aspirin ingestion. In contrast to PG\textsubscript{1}, EDRF elevates cellular levels of cGMP, rather than cyclic adenosine monophosphate (cAMP), and both of these inhibit platelet function by blocking calcium mobilization and activation of phospholipase C. Recently, it has been found that elevation of cGMP prevents breakdown of cAMP.\textsuperscript{29} This observation may explain the reported synergy between EDRF and PG\textsubscript{1}\textsubscript{2}.\textsuperscript{33}

Endothelial cell ecto-ADPase activity. Data from our laboratory indicate that an endothelial cell-associated nucleotidase system also plays an important role in regulation of platelet reactivity. This ecto-ADPase activity is aspirin-insensitive and results in catabolism of ADP by aspirin-treated endothelial cells with concomitant loss of platelet stimulatory activity in the fluid phase. Traditionally, nucleotides (eg, ATP) have been regarded mainly as intracellular energy sources. However, nucleotide release in response to cell perturbation or injury is an important (patho)physiologic event and may be of pertinence in thrombosis.\textsuperscript{34-36}

Addition of [\textsuperscript{14}C]ADP to aspirin-treated HUVEC in our

### Table 1. Thromboregulators Produced by Human Endothelial Cells

<table>
<thead>
<tr>
<th>Type</th>
<th>Example</th>
<th>Fluid Phase</th>
<th>ASA-Sensitive</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eicosanoids</td>
<td>Cyclooxygenase metabolites; PG\textsubscript{1,2}</td>
<td>Yes</td>
<td>Yes</td>
<td>Inhibit platelet reactivity by increasing cAMP</td>
</tr>
<tr>
<td>EDRF</td>
<td>EDRF/NO</td>
<td>Yes</td>
<td>No</td>
<td>Inhibit platelet reactivity by increasing cGMP</td>
</tr>
<tr>
<td>Ecto-Nucleotidases</td>
<td>ADPase(s)</td>
<td>No</td>
<td>No</td>
<td>Inhibit platelet reactivity by removing secreted ADP</td>
</tr>
</tbody>
</table>
laboratory showed the reaction sequence: ADP → AMP →
adrenalin. Therefore, in addition to removal of ADP as an
agonist, the endothelial cell ADPase results in production of
antiaggregatory ADP metabolites. It was inferred from these
in vitro observations that two situations coexist in the
microenvironment of a developing vascular lesion. Activated
platelets release adenine nucleotides, as do stimulated or
injured endothelial cells. However, the endothelial cells also
possess ecto-nucleotidases, which rapidly process extracellu-
al adenine nucleotides to adenosine, which is efficiently
taken up and further metabolized. This endothelial cell
function may be particularly important in localized areas of
high shear, where metabolism of erythrocyte-derived ADP
would be beneficial for maintenance of blood fluidity. This
regulatory function may be compromised in thrombotic
microangiopathies, resulting in increased platelet activation
by erythrocyte-derived ADP. We currently hypothesize that
ADPases serve to limit the consequences of initial vessel wall
injury.

In endothelial cells the eicosanoid system via PGI$_2$ oper-
ates in parallel with ecto-ADPase mechanisms. Eicosanoids
inhibit by elevating platelet cAMP and ecto-ADPase modu-
lates platelet recruitment. When erythrocytes are eliminated
by aspirin treatment, the ecto-ADPases still function, in
conjunction with EDRF (Table 1).

**PLATELET-ERYTHROCYTE INTERACTIONS**

The bleeding time is prolonged in anemic patients. Correc-
tion of anemia results in normalization of the bleeding time.
Traditionally this was attributed solely to ADP from dam-
aged erythrocytes. We now believe this correction is more
likely due to a metabolic property of erythrocytes, which
increases platelet activation and recruitment. This metabolic
activity is provoked by proximity of the erythrocyte to an
activated platelet.

To investigate platelet-erythrocyte interactions, a novel
two-stage in vitro procedure was developed. The system
allows the investigator to independently evaluate platelet
activation and recruitment within 1 minute of agonist addi-
tion. This capability of the system is particularly advanta-
geous because recruitment may be the more important
component of platelet reactivity, especially in thrombosis.
Experiments with combined platelet-erythrocyte suspensions
stimulated with collagen, thrombin, or ionophore A23187
showed that intact erythrocytes markedly increased platelet
serotonin release. This result occurred even when ADP was
enzymatically removed, when the system was aspirin-
treated, or both.

In addition, erythrocytes enhanced arachidonate hydroly-
sis from platelet phospholipids, as well as arachidonate
metabolism. This enhancement resulted in increased re-
lease of eicosanoids and free arachidonate from the platelet
into the fluid phase. Importantly, this erythrocyte effect on
arachidonate release provides substrate for transcellular
metabolism. An example of this transcellular metabolism
would be the capacity of a stimulated neutrophil in an
evolving thrombus to generate leukotriene B$_4$ from this
released platelet arachidonate. This reaction represents a
pathway by which erythrocytes and/or platelets can play a
role in the inflammatory response. The cellular components
of a thrombus can therefore be envisioned as forming a
"metabolic bridge," which, if appropriately studied and
comprehended, could lead to methodologies for pharmaco-
logic control of thrombus development and propagation. In
any case, we consider intact erythrocytes as prohemostatic
and prothrombotic cells.

**PLATELET-NEUTROPHIL INTERACTIONS**

Using the system mentioned above for separately studying
platelet activation and recruitment, we investigated the
effects of neutrophils on platelet responsiveness. When a
combined suspension of platelets and neutrophils was stimu-
lated with thrombin, collagen, or A23187 (1 minute), the
cell-free releasate derived therefrom contained remarkably
little released serotonin, as compared with releasates gener-
ated by platelets alone. In addition, recruiting properties of
these releasates were essentially absent, especially with
thrombin and A23187 as agonists. Thus, in direct contrast to
erthrocytes, neutrophils in contact with activated platelets
induced a market decrease in platelet reactivity. This neutro-
phil inhibitory effect on platelets was dependent on neutro-
phil number and occurred in the physiologic range. In
contrast to recently published results from two independent
laboratories, we could not show an effect of neutrophil
EDRF/NO in this system. There may indeed be at least
two mechanisms by which neutrophils inhibit platelet reactiv-
ity, i.e., EDRF-dependent and -independent. On the other
hand, it should be mentioned that in a different experimental
setting, one laboratory reported that the presence of neutro-
phils increased platelet reactivity. These observations show
that different in vitro model systems may reflect different in
vivo physiologic situations.

**PERSPECTIVES FOR THE FUTURE**

Thrombosis and atherosclerosis are multicellular pro-
cesses, the course of which may be governed by varying
degrees of cell contact and stimulation. During their develop-
ment, thrombotic and inflammatory events are biochemically
linked as part of overall host defense mechanisms. In vitro
biochemical and functional dissection of interactions be-
tween cell components of thrombi have yielded new informa-
tion relevant to the pathogenesis of both thrombotic disorders
and the inflammatory response. This information pre-
sents new therapeutic possibilities. Can endothelial cell
ADPase activity be enhanced? Is there a pharmacologic
maneuver which can increase EDRF production? Can neutro-
phil inhibition of platelet reactivity be amplified?

Therapeutic enhancement of cell-cell interactions that are
potentially antithrombotic (neutrophil inhibition of platelet
function), and blockade of those which are prothrombotic
(erythrocyte amplification of platelet reactivity) may repre-
sent novel approaches. Aspirin, the mainstay of current
antithrombotic therapy, is directed toward elimination of
thromboxane production. Experimentally, in vitro inhibitory
effects of aspirin on platelets are easily overcome by slight
increases in agonist concentration. Moreover, there is evi-
dence that thromboxane is not as strong a primary platelet
Vascular occlusion is conceptually more complicated than the
amplify responses to weak stim~li. Therefore, therapies
agonist as initially conceived,
and may serve mainly to
global," multicellular strategy toward inhibition of vascular occlusion is conceptually more complicated than the
traditional unicellular approaches, but could have more
potential and greater safety.

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