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A Journey with Tony Hugli up the Inflammatory Cascade towards the Auto-Digestion Hypothesis

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Abstract

My association with Tony Hugli, long-term editor of Immunopharmacology and International Immunopharmacology, came about by a specific and long-standing problem in inflammation research. What is the trigger mechanism of inflammation in physiological shock? This is an important clinical problem due to the high mortality associated with physiological shock. We joined forces in the search of the answer to this question for more than a decade. Our journey eventually led to development of the hypothesis that shock may be associated with pancreatic enzymes, a set of powerful digestive enzymes that are an integral part of human digestion. The digestive enzymes need to be compartmentalized in the lumen of the intestine where they break down a broad spectrum of biological molecules into their building blocks, suitable for molecular transport across the mucosal epithelium into the circulation. The mucosal epithelial barrier is the key element for compartmentalization of the digestive enzymes. But under conditions when the mucosal barrier is compromised, the fully activated digestive enzymes in the lumen of the intestine are transported into the wall of the intestine, starting an auto-digestion process. In the process several classes of mediators are generated that by themselves have inflammatory activity and upon entry into the central circulation generate the hallmarks of inflammation and eventually cause multi-organ failure. Thus, our journey led to a new hypothesis, which is potentially of fundamental importance for death by multi-organ failure. The auto-digestion hypothesis is in line with the century old observation that the intestine plays a special role on shock - indeed it is the organ for digestion. Auto-digestion may be the prize to pay for life-long nutrition.

Keywords

Auto-digestion; shock; inflammation; cytokines; leukocytes; microcirculation; pancreatic enzymes; trypsin; chymotrypsin; elastase

Introduction

My encounter with Tony Hugli was deliberate and planned. Having recognized from the short distance between the University of California San Diego and The Scripps Research Institute his pioneering work on complement, the isolation and identification of biological and pathophysiological properties of complement fragments [1], our collaboration started in the early 1990th at a luncheon. Tony's interest and generosity to consult was a natural growing

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ground to focus on an important medical problem and generate new ideas. His wit, quick reasoning and all encompassing knowledge of protein chemistry were a refreshing inspiration and source of ideas on the journey we went on to embark. We have reached a new vista on one of the most pressing problems in medicine. It has been a roller coaster with few high points and plenty of disappointments. Bioengineering, my field, and biochemistry, Tony's field, played a complimentary role; either discipline by itself would have been inadequate to take us to the level of understanding we have reached today.

The Inflammatory Cascade

The problem that brought us together was the phenomenon referred to as *inflammation*. By 1985, an extensive body of preclinical, experimental evidence had accumulated, to indicate that many cardiovascular diseases were accompanied by markers for inflammation, such as elevated endothelial permeability, attachment of leukocytes to the endothelium of postcapillary venules, obstruction of capillaries by leukocytes as well as numerous biochemical markers [2]. Several interventions against individual steps in the inflammatory cascade were subject to preclinical testing and there was new hope that interventions against individual steps in the inflammatory cascade may have a beneficial clinical outcome.

By the turn of the century clinical evidence in a variety of patient groups started to support the hypothesis that inflammatory markers serve as statistically significant predictors for clinical outcome. Inflammation took center stage as a key event in the progression of important human diseases, e.g. atherosclerosis, cardiac and cerebral ischemia, and chronic degenerative diseases, and many others [3,4,5,6,7]. This list continues to rapidly grow with a continuous stream of publications describing clinical trials that demonstrate the presence of inflammatory markers even in diseases that in the past have not been associated with inflammation (e.g. arterial hypertension, cancer, aging) [8,9,10,11,12,13,14,15,16].

But most of the interventions against the inflammation that were proposed had one common characteristic; they were designed to block individual steps in the inflammatory cascade irrespective of the particular origin of inflammation, in fact in many cases the design of the intervention was ignorant of the actual trigger mechanism for the inflammatory cascade. Interventions to block oxygen free radical production, to enhance the level of depleted nitric oxide, to block membrane protein adhesion between leukocytes and endothelium (e.g. P-, E-, or L-selectins, integrins, ICAMs), or to block matrix metalloproteinases, to name a few, were designed without targeting or even knowledge of the trigger mechanism(s) of the inflammation. The purported causes of the inflammation were speculative and unconfirmed in many cases.

Traditional Interventions against the Inflammatory Cascade

The deficiency in our understanding of the trigger mechanisms for inflammation was evident when we tested blood samples from individuals with risk factors for cardiovascular disease [17] and even more so with experimental forms of physiological shock [18]. Such plasma samples exhibit significant pro-inflammatory activity. We wondered, what is the effectiveness of blocking the inflammatory cascade in an individual in which pro-inflammatory mediators in the plasma are detectable? It is an exercise in contradiction. To be effective against the inflammatory cascade, it is necessary to identify and then interfere the very trigger mechanism for the inflammation; blockade of later steps in the inflammatory cascade seems futile.

In fact, we have to recognize that the inflammatory cascade serves a lifetime as tissue *repair mechanism* after injury. It is capable to lead to a *resolution of inflammation*, i.e. formation of a repaired tissue, either capable to carry out the original organ functions or just serving as connective tissue in a scar. We wondered whether blockade of individual steps in the inflammatory cascade may not also impair or block the tissue repair mechanisms and therefore

the resolution of the inflammation. We were therefore not surprised that clinically effective treatments by use of several of these interventions against selected inflammatory steps mentioned above were *not* coming forward in the literature. It became apparent that there is a need to develop an alternative approach to interfere with the inflammatory cascade in many human diseases.

Inflammation in Physiological Shock

Nowhere is the lack of firm knowledge about the trigger mechanisms more visible than in the severe forms of inflammation encountered in physiological shock - a condition with extraordinary high mortality. Shock is accompanied by high levels of inflammatory mediators in plasma and in lymph fluid. In experimental forms of hemorrhagic shock we detect significantly elevated levels of inflammatory markers already within one hour after central blood pressure reduction [19,20]. The markers can be detected by exposure of plasma to naïve leukocytes from a donor animal. These inflammatory mediators have been reported repeatedly in the past and have received various designations, e.g. leukocyte activating factor, clastogenic factor, myocardial depressing factor, T-cell proliferation depressing factor, and others [21]. None of these designations fully embrace the spectrum of activity that is associated with plasma from individuals with physiological forms of shock. In general, shock plasma depresses cell functions irrespective of the particular cell type under investigation. In-vivo the appearance of inflammatory mediators in plasma is accompanied by multi-organ failure often in relatively rapid succession following the initial insult that precipitates the shock.

Inflammatory Mediators

Thus, we were confronted by a fundamental question: What are the biochemical mediator(s) that may be responsible for the depression of cell function in shock? The literature pointed towards mediators, such as endotoxin, cytokines, platelet activating factors, and complement [22,23,24,25,26,27]. But several attempts could not confirm any of them in a conclusive fashion [28], especially in clinical trials. Yet, antibodies against complement 5a were effective in improving the hemodynamic complications associated with endotoxic shock [29]. The blood samples we collected from rats after hemorrhagic shock contained no significant levels of endotoxin, no detectable levels of cytokines, such as TNF α , and in repeated attempts we could not demonstrate that complement fragments were responsible for the powerful leukocyte activation produced by shock plasma [19].

Yet, when the plasma or lymph samples [20] from shock animals was exposed to naïve leukocytes they exhibit tell-tale sign of inflammation and cell activation, including pseudopod projection, oxygen free radical formation, degranulation and membrane adhesion receptors. Thus it was apparent that any attempt to reduce the level of inflammation in shock would need to either achieve this in spite of the stimulation caused by the plasma or would have to involve a process that interferes with the source of these inflammatory mediators in the first place.

My attempts to convince Tony to subject our shock plasma samples, which did contain the inflammatory mediators, to gel filtration or reverse phase high pressure liquid chromatography separation and eventual mass spec identification ran into significant problems. Even when we collected plasma samples from several rats subjected to hemorrhagic shock, at one time reaching a volume of close to 50 ml, the inflammatory activity in the plasma disappeared rapidly after one or at most two passages over the biochemical separation columns. Tony emphasized that although the plasma contained a powerful inflammatory mediator it was likely in low concentrations and rapidly eliminated on any separation column. Our review of the literature and consultation with investigators who had previously attempted to identify specific biochemical species from shock plasma confirmed that we were working with a molecular mass of inflammatory mediators inadequate for conclusive biochemical identification. Unless

prior knowledge about the biochemical nature of these mediators exists or tools are available to aid in their identification, little progress was possible. We, and others before us, could not identify an approach to pinpoint the inflammatory mediators in shock. Consequently all proposed shock treatments in the literature were designed without firm knowledge about the origin of inflammation in shock! It was not surprising to us that few effective treatments had been developed against the lethal course of shock in patients. We needed a fresh approach.

Tracking Inflammatory Mediators

This opportunity arose when Professor Alan Lefer of Thomas Jefferson University spend a sabbatical in my laboratory in 1995. He shared his decade long experience in attempting to isolate an inflammatory mediator encountered in shock which he had designated as the “Myocardial Depressing Factor” [30,31]. We determined that the same plasma samples generate the characteristic depression of cardiac muscle contraction as well as the typical activation in circulating leukocytes. Professor Lefer and Dr. Erik Kistler suggested to look for a possible source of inflammatory mediators by use of selected organ homogenates. While this approach does not directly identify the actual inflammatory mediator in plasma, it may still be helpful for this problem since it may suggest which organ or cells may be a source of mediators under conditions of shock.

Pancreatic Digestive Enzymes as Source for Inflammatory Mediators

Dr. Kistler quickly identified the pancreas as a major source for inflammatory mediators [32] in line with the observations of the pancreas as a potential source for the myocardial depressing factor [32]. Kistler demonstrated that homogenates of the pancreas from different species stimulate not only typical signs of inflammation, like leukocyte activation in-vitro or leukocyte adhesion in-vivo to the endothelium of postcapillary venules, but also directly caused cytotoxicity in-vivo [32]. Homogenates of pancreas cause high mortality. Among the four general classes of digestive enzymes of the pancreas (proteases, amylases, lipases and nucleases) the major cytotoxic factors can be generated by means of serine protease (e.g. trypsin, chymotrypsin, elastase) and lipases [33]. Addition of trypsin, chymotrypsin, elastase or lipase to organ homogenates that by themselves produce only low inflammatory activity (e.g. the heart, liver, brain, spleen, kidney) causes generation of levels as high as in pancreatic homogenates, to the point of frank cytotoxicity [33].

Inflammatory activity in these organ homogenates is generated not only by the mixture of tissue and digestive enzyme, but also by low molecular filtrates (<5000 Dalton) without the higher molecular weight pancreatic enzymes [34]. This evidence suggests that the inflammatory activity is caused at least in part by products derived from the pancreatic enzyme activity.

The evidence opened two new major possibilities. On one hand Tony Hugli could start a systematic analysis of the particular biochemical species involved, but this time derived from a large supply of pancreatic homogenates derived from the slaughter-house. On the other hand, we could start to examine by an independent approach the hypothesis that pancreatic digestive enzymes may be involved in the inflammatory process in shock.

The Biochemistry of Inflammatory Mediators

While in the past a number of inflammatory mediators have been detected in plasma from shock victims (endotoxin, platelet activating factor, cytokines, complement, coagulation and fibrinolytic fragments, arachidonic acid products like leukotrienes, thrombin, oxidized products, to name just some) [35], no evidence exists that any of these mediators are the primary source of the inflammatory or cytotoxic reactions. Proof is missing that depletion of any one of them serves to stop the inflammation in shock, i.e., it remains to be determined what is the

pathophysiologically significant biochemical species that is responsible for inflammation and cell activation in multi-organ failure.

Tony's analysis of pancreatic digests showed that both high- and low-molecular weight species were involved in the inflammatory activity [32]. Separation of pancreatic organ homogenates with HPLC at different concentrations of acetonitrile concentrations to separate hydrophilic and hydrophobic species yielded a mixed picture of both protein- and lipid-derived species [35]. Separation of pancreatic homogenates into aqueous and lipid fraction with chloroform/methanol yielded two fractions with dichotomous properties: in-vivo both aqueous and lipid fraction caused high mortality, yet in-vitro only the lipid fraction caused significant degranulation of neutrophils [36]. The mechanism is currently unknown.

The Intestine as Source of Inflammatory Mediators

Pancreatic enzymes are released into the lumen of the intestine as part of normal digestion. An ischemic intestine is associated with elevated permeability [37,38,39,40,41] and therefore the usual compartmentalization of digestive enzymes in the lumen of the intestine may no longer be preserved. The question arises whether products generated during inadvertent entry of digestive enzymes into the wall of the intestine may cause an inflammatory reaction. While normal intestinal tissue by itself generates minimal levels of inflammatory mediators, intestine after ischemia generates significant inflammatory mediators [42]. Highly cytotoxic inflammatory mediators may be generated in the intestinal wall by the mixture of digestive enzymes from the lumen of the intestine or even by individual proteases (e.g. trypsin, chymotrypsin, elastase) [43]. In fact, in the rat, regular commercial chow will generate a powerful cytotoxic mediator if incubated with the mixture of pancreatic digestive enzymes [43]. This is a surprising result. Food may contain cytotoxic components once it enters into the intestine and is being digested by pancreatic enzymes? This is an important issue that requires a great deal more investigations.

Blockade of Digestive Enzymes in the Lumen of the Intestine

In the meantime, the question arises whether blockade of the pancreatic digestive enzymes in the lumen of the intestine may serve to reduce the production of inflammatory mediators in shock. Indeed, blockade of the pancreatic digestive enzymes in the lumen of the ileum serves to reduce the level of inflammatory mediators in shock produced by occlusion of the superior mesentery artery [44], irrespective of the particular choice of the inhibitors [45], in hemorrhagic shock [46], even after some delay in the enzyme blockade [47], or in endotoxic shock [48]. The protection provided by the protease inhibition is not enhanced by supplementation with oxygen free radical blockers [49]. Blockade of digestive enzymes in the lumen of the intestine during ischemia serves to prevent formation of inflammation in remote organs [50]. Blockade of digestive enzymes in the lumen of the intestine also reduces the need for fluid required to maintain blood pressure after a severe form of ischemia and eventually also reduces the level of inflammatory mediators in the plasma [51].

Conclusions

For most living creatures, shock and multiorgan failure is a problem second-to-none. Unless death is caused by an abrupt event (e.g. trauma, fibrillation), multi-organ failure is likely to play a central role when the circulation come to a stand-still. Understanding the origin of the inflammatory cascade that leads to the progressive failure of vital organs is the basis for rational and optimal treatment. It has become evident that there are several classes of inflammatory mediators which in the simplest of all analysis need to be divided into two classes: (a) the group of inflammatory mediators that are actually involved in breakdown of the tissue after an insult (e.g. protein and lipid fragments generated by pancreatic digestive enzymes, complements),

and (b) the inflammatory mediators — perhaps better designated as *signaling or repair molecules* — that are part of the resolution associated with inflammation (e.g. cytokines). The evidence for the first group has come from studies of organ homogenates generated in the presence of digestive enzymes and from studies with blockade of digestive enzymes as an in-vivo intervention. While the evidence for the second group is already well documented, the evidence for the first group is new and less well understood. Clinical interventions need to be directed at the first group of mediators. But for now a new hypothesis for the origin of inflammation in shock has been born: the *Auto-digestion hypothesis* (Figure 1) [52], i.e. a breakdown of a variety of autologous molecules by one's own digestive enzymes. So far, the evidence supporting this hypothesis is derived from preclinical studies [53]. The evidence in man is still missing but the idea will spark clinical studies. We celebrate Tony Hugli and hope he will continue to be an integral part of the effort.

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1. . Schematic diagram of the *Auto-Digestion* hypothesis

In the normal intestine (left) the epithelial mucosal barrier prevents escape of pancreatic digestive enzymes to limit digestive activity in the lumen of the intestine. Digestive products (e.g. amino acids, nucleotides, ions, carbohydrates, lipids) are transported active and passive across the epithelial barrier with minimal entry of digestive enzymes. In shock (right), the epithelial barrier properties become compromised and permit entry of fully activated digestive enzymes into the intestinal wall, a process that starts autodigestion and generation of lipid and protein fragments from intestinal tissue that are pro-inflammatory and even cytotoxic. They are transported via the intestinal veins via the portal venous system into the hepatic circulation, via the intestinal lymphatics into mesentery lymph nodes, and directly via the intestinal wall into the peritoneal cavity.