The Activation of Platelet Aggregation by Human Cholangiocarcinoma Cells is Mediated Through Thrombin Receptor

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Abstract

Tumor cell induced platelet aggregation (TCIPA) played an importance role in early state of thrombosis in cancer patients. In addition, TCIPA was recognized as one important step in metastatic cascade. Cholangiocarcinoma, one of the most common cancers in the north-eastern part of Thailand, associated with thrombosis was reported. The authors investigated the effects of cholangiocarcinoma cells on platelet function as measured by platelet aggregation. Primary human chlolangiocarcinoma (HuCCA) cells were established in our laboratory. Cells were cultured as standard techniques and grown to confluence until used, after which cells were replaced with fresh medium (Dulbeco Modified Eargle's Medium, DMEM) without serum for 24, 48 and 72 h. Then, the conditioned medium (CM) was collected. CM (24, 48 and 72 h) from HuCCA failed to induce platelet aggregation, whereas, HuCCA pellets induced platelet aggregation and potentiated platelet aggregation induced by submaximal concentration of thrombin. Interestingly, platelet aggregation induced by HuCCA was inhibited by hirudin (thrombin receptor antagonist; 10, 20 and 40 U) in a dose dependent manner. Thus, cholangiocarcinoma cells can induce platelet aggregation in a direct tumor cell-platelet contact *via* thrombin receptor. Therefore, the use of antiplatelet agents especially *via* thrombin receptors may help to prevent TCIPA or metastasis by CCA.

Key word : Chlolangiocarcinoma, Thrombosis, Platelets, Thrombin, Receptor, Signalling

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The relationship between malignancy and thrombosis has long been recognized as a major cause of morbidity and mortality in cancer patients (1). The cause of these hemostatic disorders are strongly linked to the capacity of tumor cells to interact with components of the blood. The platelet activation system is one pathway that has been consistently implicated in the pathogenesis of thrombosis in cancer patients(2). Tumor cell induced platelet aggregation (TCIPA) has been reported in various cancers, such as mucin-secreting adrenocarcinomas, myelo-proliferative disorders, acute promyelocytic leukemia or brain tumors(3). Cholangio-carcinoma (CCA) is a primary intrahepatic cancer of the biliary epithelium. It is a rare disease in Western countries but is common in the north-eastern part of Thailand, where the prevalence of liver fluke infection is the highest(4). CCA seems to have a very high rate, probably the highest in Asia. The association between CCA and thrombosis has been reported in many studies(5-7). However, the mechanism by which CCA facilitates platelet function is unknown. Thus, the study of interaction between CCA and platelets will help to gain a better understanding of the pathophysiology of TCIPA leading to rationale use of drugs such as antiplatelet drugs. In this study, the experiments were designed to explore the effects of human cholangiocarcinoma (HuCCA) cell culture on platelet functions as measured by platelet aggregation. The cellular mechanisms by which TCIPA will be also elucidated.

MATERIAL AND METHOD

Subjects

The study was performed on 25 healthy volunteers. There were 12 females, 13 males and the age range was 25-55 years. All subjects had not ingested aspirin (ASA), any other NSAIDs, or antiplatelet drugs for at least 2 weeks before beginning the study and they fasted overnight.

HuCCA cell culture

Primary HuCCA cells were established in our laboratory. Cells were cultured in T-75 flasks with Dulbecq Modified Eargle's Medium (DMEM) containing 15 per cent fetal bovine serum (FBS), 100 unit/ml penicillin G, 100 μg/ml streptomycin and grown to confluence until used, after which time cells were replaced with fresh medium (DMEM) without serum for 24, 48 and 72 hours. Then, the conditioned medium (CM) was collected. To study the whole cell effect, HuCCA cells were grown to confluence and extracted using trypsinization. Cells were resuspended in DMEM to yield a concentration of 10^7 cells/ml.

Platelet aggregation study

Blood samples were obtained by venipuncture and gently mixed with 3.8 per cent sodium citrate (1:9 by volume) or 8 U/ml heparin as an anticoagulant. Platelet rich plasma (PRP) was prepared by centrifugation at 250 g for 15 minutes at room temperature. Platelet counts were determined using a counting chamber. The concentration of platelets in PRP was $5.0\pm1.0 \times 10^8$ cells/ml. Platelet aggregation was determined by Born's technique using an aggregometer(8). The aggregation study was done within 2-3 h of blood collection.

The state of platelet aggregation was categorized into one of three states (hyper-, normal- and dis-aggregation) before the experiment. Hyper-aggregated platelet state was defined for the platelets that were highly responsive to the lower concentration of aggregating agents (eg. 1 μM adrenaline) while the dis-aggregated platelet state was defined as platelets that responded slightly to the higher concentration of aggregating agents (eg. 25 μM adrenaline). Normal aggregated platelet state was defined as platelets that responded slightly or highly depending on the concentration of aggregating agent (e.g. slight response to 1 μM adrenaline and high response to 25 μM adrenaline).

The effect of HuCCA on platelet aggregation study was done by using 850 μl of PRP and recorded for 7 minutes after adding DMEM (150 μl, control), CM (at 24, 48 and 72 h, 150 μl), HuCCA cell suspensions (1.5×10^7 cells/150 μl) alone or HuCCA cell suspensions (1.5×10^7 cells/150 μl) plus hirudin (10, 20 or 40 U). Each experiment was repeated twice. Platelet aggregation was evaluated by comparing the amplitudes of the aggregation curves.

Statistical analysis

Results are shown as mean ± S.E. mean from duplicate determinations (samples) from at least three subjects on separate experimental days (n=6). Student's paired or unpaired *t*-tests, as appropriate, were used to determine the significance of differences between means and p-values of less than 0.05 were taken as statistically significant.

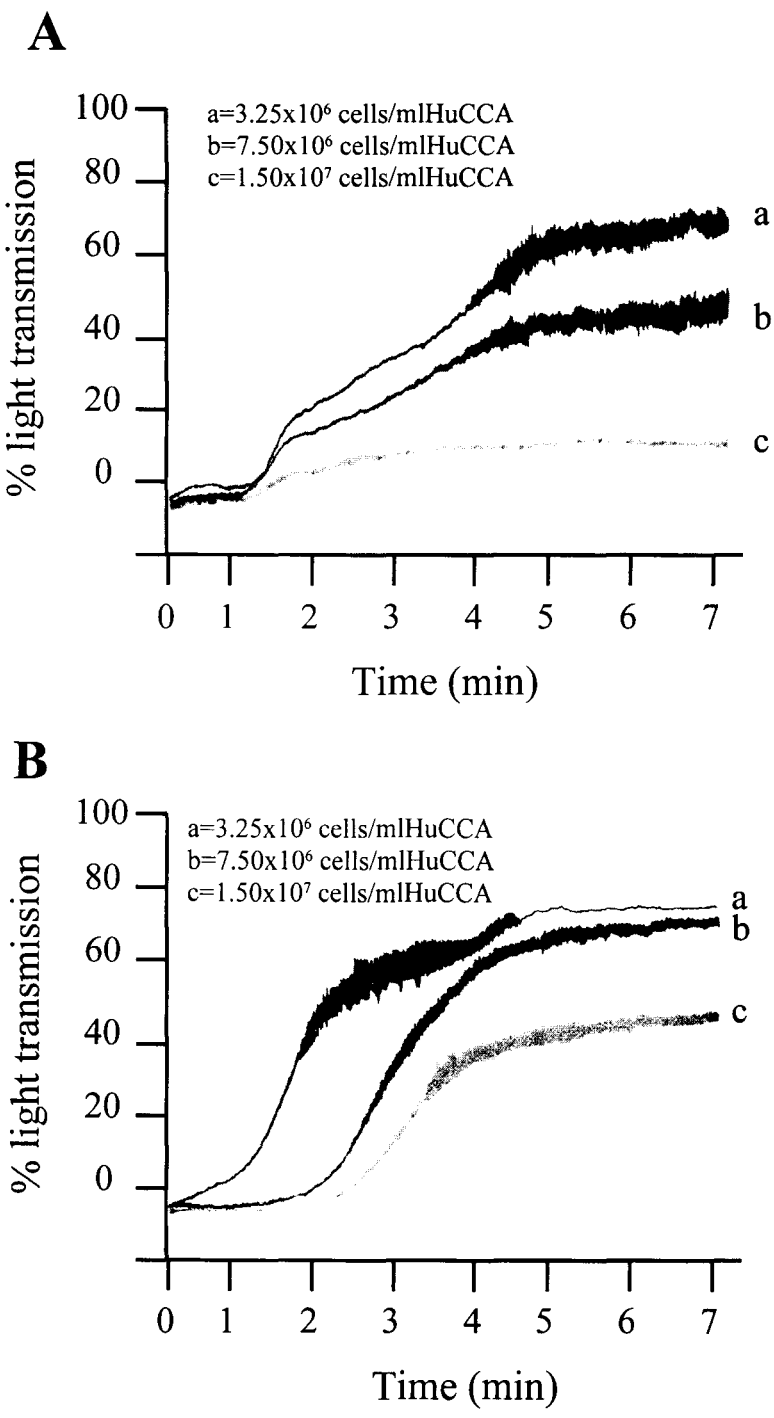

Fig. 1. **Effects of different concentrations of HuCCA on platelet aggregation. Panel A represents tracing of HuCCA induced platelet aggregation in sPRP. Panel B represents tracing of HuCCA induced platelet aggregation in hPRP.**

Materials

Adrenaline, ADP and thrombin were purchased from Sigma® (USA). DMEM and FBS was purchased from Hyclone® (Utah, USA). Hirudin was obtained from Feinchemic GmbH Sebnitz (Germany).

RESULTS

Effects of HuCCA on platelet aggregation

HuCCA cell suspension induced platelet aggregation in PRP while CM 24, 48 and 72 hours had no effect on platelet aggregation. The potency of HuCCA cells to induce platelet aggregation was different depending on the concentration of cell suspension (Fig. 1). The various batches of HuCCA cell culture (CL-2 CL-6 and CL-19) gave a similar effect on platelet aggregation (Fig. 2).

However, when using a different anticoagulant in the stage of preparation of PRP, the ability of HuCCA to induce platelet aggregation was different in each subject. HuCCA induced platelet aggregation of all subjects in heparinized PRP (hPRP) while some subjects in sodium citrated PRP (sPRP) did not respond (Fig. 3).

Interestingly, the effects of HuCCA induced platelet aggregation in sPRP were different in each group in the platelet status of the subjects. HuCCA cells initated platelet aggregation in six of ten subjects in the hyper aggregation platelet state (73.04 ± 5.73 and 0% for HUCCA and DMEM, respectively) but HuCCA cells did not induce aggregation of platelets in the normal and dis-aggregated platelet state (Fig. 3A).

Effects of HuCCA plus thrombin on platelet aggregation

HuCCA potentiated the aggregation of platelets induced by submaximum concentration of thrombin in subjects that HuCCA alone did not induce platelet aggregation. The potency of HuCCA to synergize thrombin-stimulating platelet was varied in each group of the platelet status of the subjects (Fig. 4). In the hyper-aggregated state, the maximum aggregation induced by thrombin was 13.62 ± 1.5 and 63.75 ± 7.7 per cent for thrombin alone and HuCCA plus thrombin, respectively. In the normal aggregated state, the maximum aggregation induced by thrombin was 13.43 ± 3.00 and 47.41 ± 8.75 per cent for thrombin alone and HuCCA plus thrombin, respectively. In the dis-aggregated state, the maximum aggregation induced by thrombin was 13.52 ± 2.92 and 44.71 ± 15.79 per cent for thrombin alone and HuCCA plus thrombin, respectively (Fig. 4).

Effects of hirudin on HuCCA induced platelet aggregation

Hirudin, a thrombin antagonist, had different effects on HuCCA induced platelet aggregation in the sPRP and hPRP groups. Hirudin did not inhibit HuCCA induced platelet aggregation in sPRP (Fig. 5). Whereas, HuCCA induced platelet aggregation in hPRP was inhibited by hirudin in a dose dependent manner (Fig. 6). The inhibition of HuCCA induced platelet aggregation by hirudin was significant at 10 U of hirudin (Fig. 6, $p < 0.05$).

DISCUSSION

The authors have shown that HuCCA induced platelet aggregation while conditioned medium failed to aggregate platelets in PRP. The results suggested that tumor cell-platelet contact might even be necessary for HuCCA induced platelet aggregation (TCIPA). Furthermore, HuCCA may potentially play important roles in activation of platelets, which would also explain the pathogenesis of thrombosis in CCA patients. The results were similar to other investigators who reported that various tumor cells play a role in platelet aggregation *in vitro*, such as those with mucin-secreting adrenocarcinomas, myeloproliferative disorders, acute promyelocytic leukemia or brain tumors[9]. In addition, TCIPA was considered to be an important event in the process of hematogenous metastasis[10]. Several studies have shown the importance of tumor cell-platelet interaction during hematogenous metastasis[11]. Platelets may stabilize the attachment of tumor cells to endothelial cells and subendothelial matrix[12,13]. Furthermore, activated platelets may also protect adherent tumor cells from natural killer cells and reduce the cytotoxic effects of macrophage-derived tumor necrosis factor-α (TNF-α) on tumor cells at the place of primary or metastatic growth[14]. Therefore, HuCCA induced platelet aggregation may be involved in the process of metastasis in cholangiocarcinoma patients.

Interestingly, the effects of HuCCA induced platelet aggregation was varied when using different anticoagulant for the preparation of PRP. HuCCA could induce platelet aggregation in some

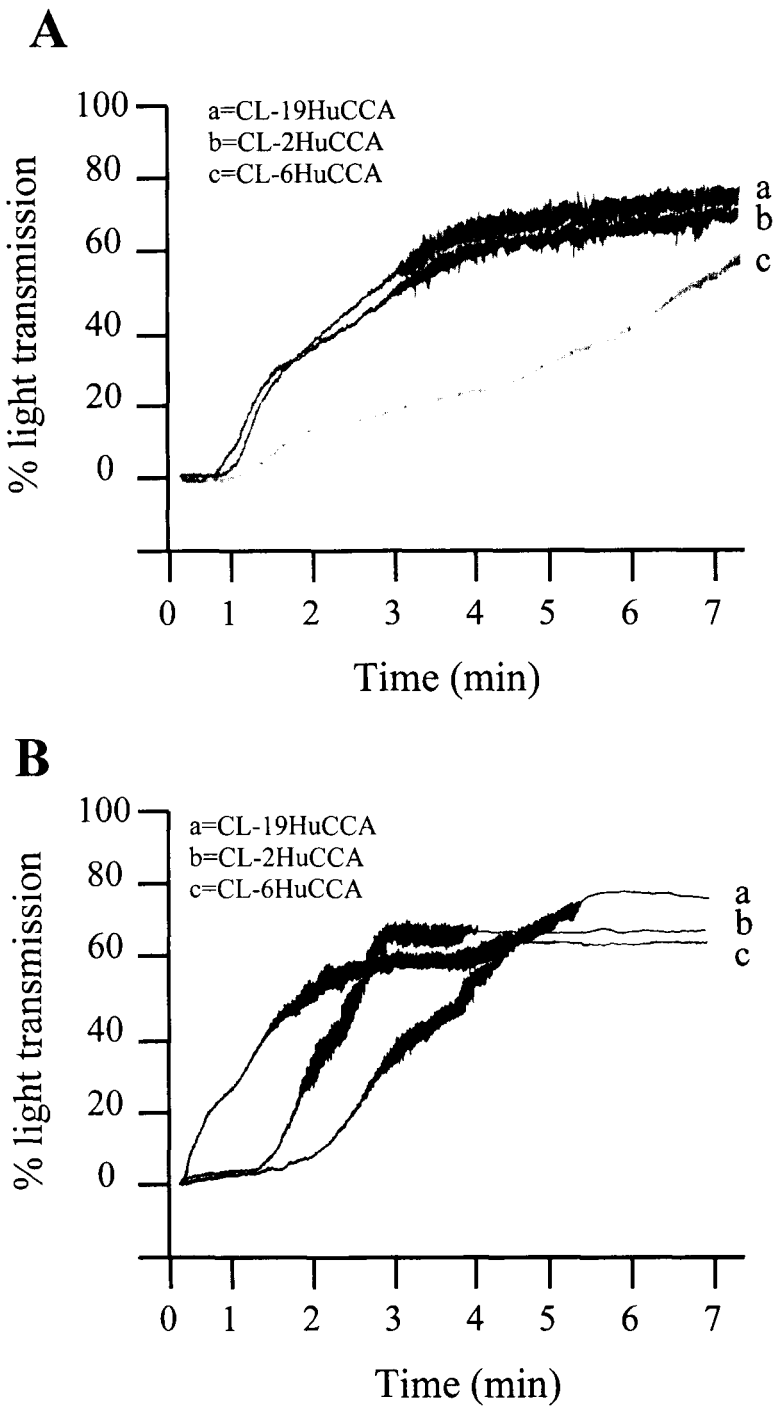

Fig. 2. **Effects of different batches of HuCCA on platelet aggregation. Panel A represents tracing of HuCCA induced platelet aggregation in sPRP. Panel B represents tracing of HuCCA induced platelet aggregation in hPRP.**

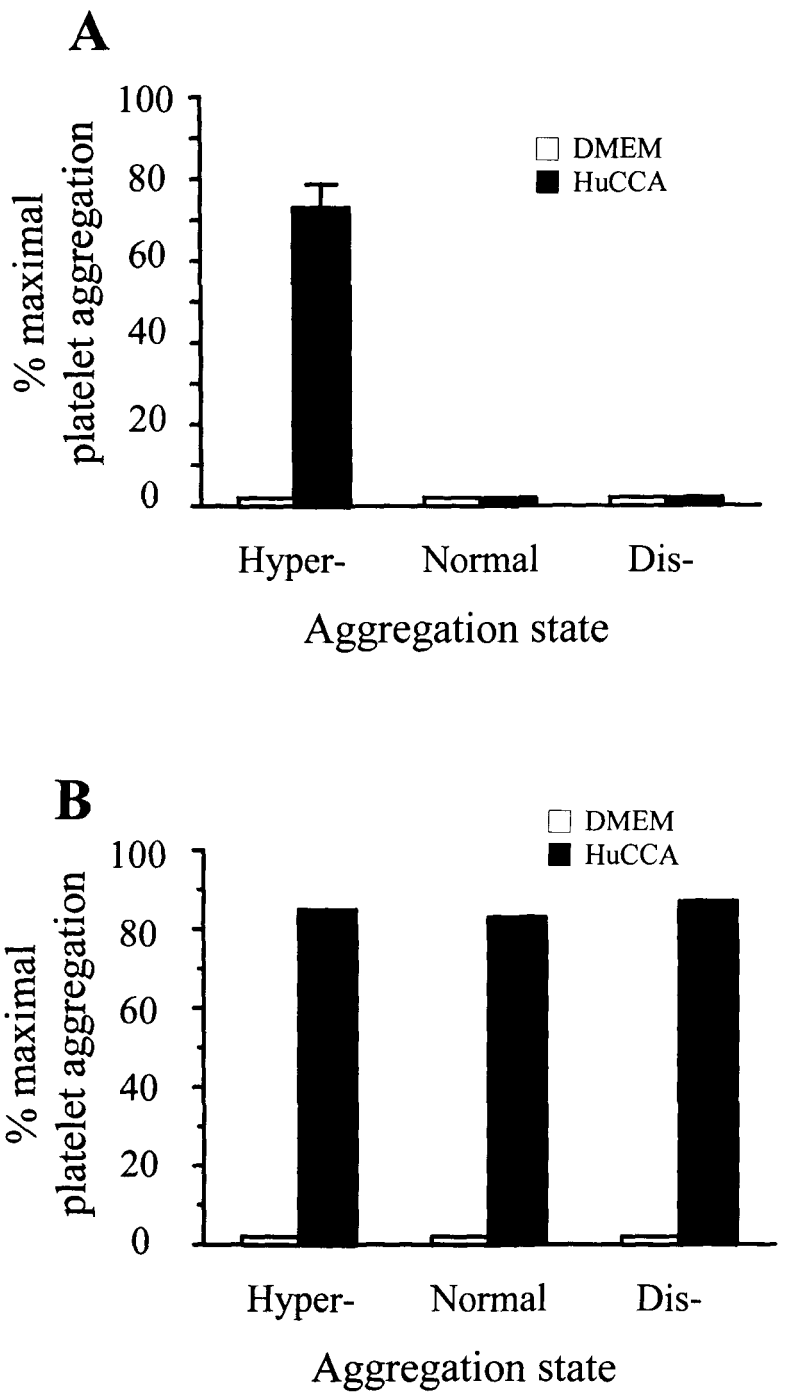

Fig. 3. **The different effects of HuCCA induced platelet aggregation when using different anticoagulants for PRP preparation. Panel A shows effects of HuCCA induced platelet aggregation in sodium citrated PRP (sPRP). Panel B shows effects of HuCCA induced platelet aggregation in heparinized PRP (hPRP).**

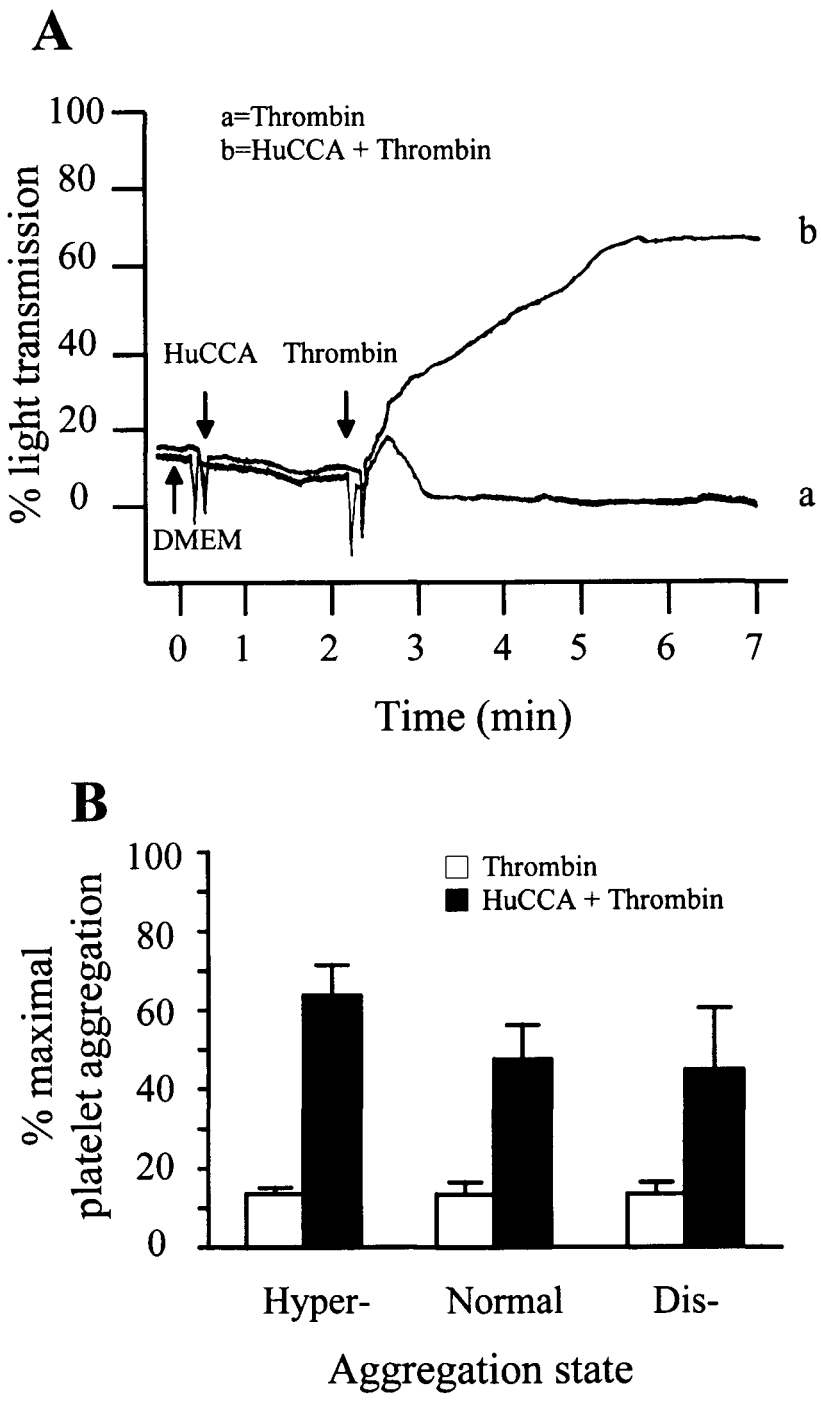

Fig. 4. **Effects of HuCCA potentiated platelet aggregation induced by submaximal concentration of thrombin. Panel A represents tracing of platelet aggregation induced by thrombin alone or HuCCA plus thrombin. Panel B shows per cent maximum of platelet aggregation induced by thrombin alone or HuCCA plus thrombin.**

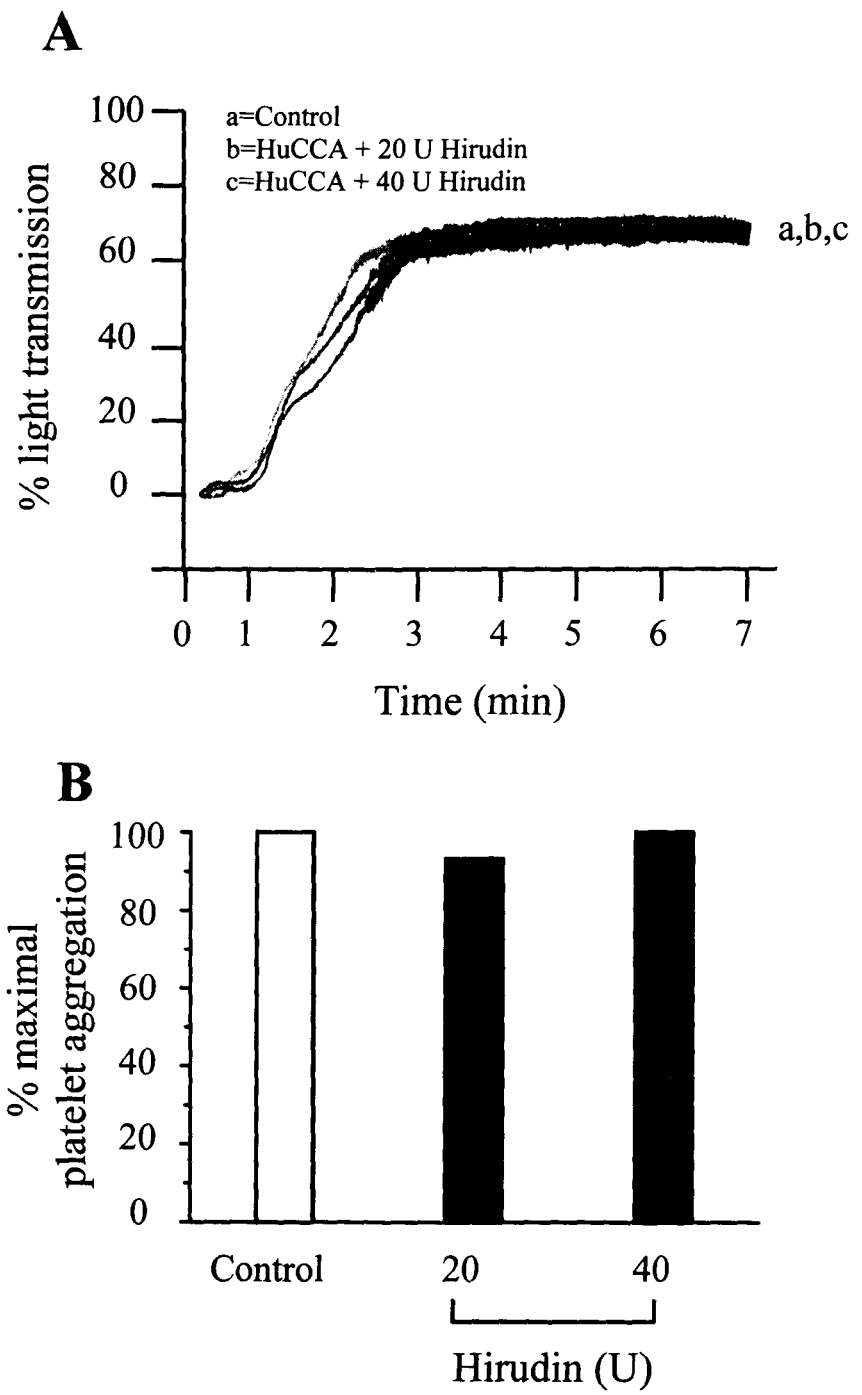

Fig. 5. **Effects of hirudin on HuCCA induced platelet aggregation in sPRP. Panel A represents tracing of platelet aggregation induced by HuCCA plus hirudin (10, 20 and 40 U). Panel B shows the effects of platelet aggregation induced by HuCCA plus hirudin (10, 20 and 40 U).**

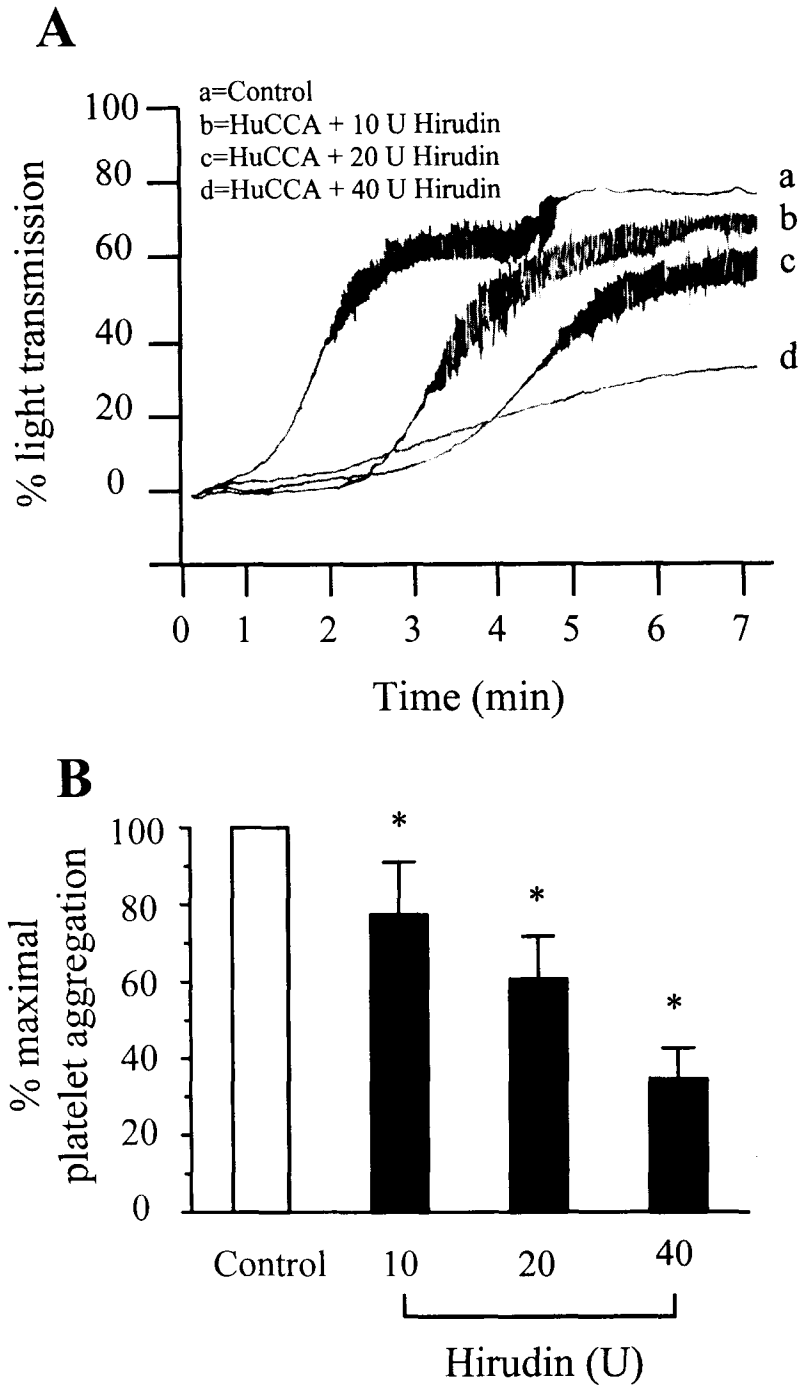

Fig. 6. Effects of hirudin on HuCCA induced platelet aggregation in hPRP. Panel A represents tracing of platelet aggregation induced by HuCCA plus hirudin (10, 20 and 40 U). Panel B shows the effects of platelet aggregation induced by HuCCA plus hirudin (10, 20 and 40 U). *$p < 0.05$ when compared to control.

subjects when using sodium citrate as an anticoagulant, whereas, platelet aggregation was induced by HuCCA in all subjects when using heparin as an anticoagulant. As a different mechanism of anticoagulant, sodium citrate formed a complex with calcium while heparin formed a complex with antithrombin III and increased its efficacy[15]. Thus, it can be postulated that the high concentrations of calcium required for the mechanism of HuCCA induced platelet aggregation when sodium citrate was used as an anticoagulant (sPRP). These findings were supported by Katagiri et al, who reported that tumor cells no longer stimulated platelets if the system was depleted of divalent ions[16]. For some subjects in whom HuCCA induced platelet aggregation in sPRP, it can be deduced explained that this group of subjects had a high concentration of calcium in their plasma. Thus, the constant use of 3.8 per cent sodium citrate solution 1:9 by volume may not be enough to catch all the calcium in the plasma in these subjects who used sPRP.

Interestingly, HuCCA potentiated the effects of submaximal concentrations of thrombin and caused complete platelet aggregation in sPRP groups in which HuCCA could not induce platelet aggregation. The ability of HuCCA potentiated platelet aggregation varies in the platelet status of each group. In a hyper-aggregated platelet state, tumor cells had a higher increase of the maximum of aggregations of platelets than in the normal and disaggregation platelet state (Fig. 4). These data indicated that the platelet status of each subject probably accounted for the potency of HuCCA induced platelet aggregation. Thus, monitoring the platelet activity may be necessary for cancer patients.

The mechanisms of HuCCA induced platelet aggregation in this experiment were also evaluated by using hirudin, a polypeptide specific inhibitor of thrombin. Hirudin did not inhibit HuCCA induced platelet aggregation in the sPRP groups. In contrast, hirudin inhibited HuCCA induced platelet aggregation in the hPRP group in a dose dependent manner. These results indicated that HuCCA induced platelet aggregation in hPRP was mediated through a thrombin receptor. However, the question remains whether thrombin mediated HuCCA induced platelet aggregation in sPRP. In the case of thrombin mediated action, the reaction required a dramatic increase in the cytosolic free Ca^{2+} cocentration[17]. Therefore, Ca^{2+} release alone from the dense tubular system (intracellular calcium) in sPRP is not enough for thrombin reaction. Both intracellular calcium and Ca^{2+} influx across the plasma membrance (extracellular calcium) are required to support this reaction. In the case of a reaction unrelated to thrombin, it may be modified at the level of receptors and needs to be investigated.

In hPRP, however, the response of HuCCA induced platelet aggregation was not completely blocked by hirudin although the maximum concentration of hirudin (40 U) was used (about 60% inhibition, Fig. 6). This suggested that there were other signalling pathways, besides thrombin, in HuCCA induced platelet aggregation.

In conclusion, the authors found that HuCCA was able to induce platelet aggregation in a direct tumor cell-platelet contact. The ability of HuCCA to activate platelet aggregation was varied (in each subject) according to the patterns of platelet activity. Thrombin and calcium play an important role in the mechanism of HuCCA induced platelet aggregation which would also explain the pathogenesis of thrombosis and metastasis in CCA patients. However, other mechanisms by which HuCCA induced platelet aggregation remain to be elucidated.

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เซลล์เพาะเลี้ยงมะเร็งท่อทางเดินน้ำดีของคนกระตุ้นการจับกลุ่มของเกร็ดเลือดโดยผ่านทาง thrombin receptor

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การกระตุ้นการจับกลุ่มของเกร็ดเลือดจากเซลล์มะเร็ง (Tumor cell induced platelet aggregation, TCIPA) จัดเป็นกลไกที่สำคัญอันหนึ่งในการเกิดลิ่มเลือด (thrombosis) ในผู้ป่วยมะเร็ง รวมไปถึงการแพร่กระจายของเซลล์มะเร็ง มะเร็งท่อทางเดินน้ำดี (Cholangiocarcinoma, CCA) จัดอยู่ในกลุ่มมะเร็งที่พบมากสุดในภาคตะวันออกเฉียงเหนือของประเทศไทย และมีรายงานการเกิด thrombosis ในบางราย การศึกษานี้จะทำการศึกษาผลของ CCA ต่อการทำหน้าที่ของเกร็ดเลือด เซลล์เพาะเลี้ยงมะเร็งท่อทางเดินน้ำดีของคน (HuCCA) ถูกนำมาเลี้ยงตามวิธีการมาตรฐาน และนำมาใช้เมื่อเซลล์โตเต็มที่ โดยนำน้ำเลี้ยงเซลล์ที่ทิ้งไว้ 24, 48 และ 72 ชั่วโมง (conditioned medium, CM) และส่วนของเซลล์ที่แยกออกมา (HuCCA pellets) มาศึกษาผลต่อการจับกลุ่มของเกร็ดเลือด พบว่า CM (ทั้ง 24, 48 และ 72 ชั่วโมง) ไม่มีผลต่อการจับกลุ่มของเกร็ดเลือด ในขณะที่ HuCCA pellets สามารถกระตุ้นการจับกลุ่มของเกร็ดเลือดได้ โดยสามารถยับยั้งการกระตุ้นดังกล่าวได้จากการใช้ hirudin (thrombin receptor antagonist; 10, 20, 40 U) ได้อย่างมีนัยสำคัญทางสถิติ จากการศึกษาวิจัยบ่งชี้ว่าเซลล์มะเร็งท่อทางเดินน้ำดี สามารถกระตุ้นการจับกลุ่มของเกร็ดเลือดได้โดยผ่านทาง thrombin receptor ดังนั้นการใช้ยาต้านการจับกลุ่มของเกร็ดเลือดโดยเฉพาะที่ออกฤทธิ์ผ่าน thrombin receptor อาจช่วยป้องกันการเกิดลิ่มเลือดหรือการแพร่กระจายของเซลล์มะเร็งท่อทางเดินน้ำดีได้

คำสำคัญ : มะเร็งท่อทางเดินน้ำดี, ลิ่มเลือด, เกร็ดเลือด, ธรอมบิน, รีเซปเตอร์, กลไกการออกฤทธิ์

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