ACTIVATION MARKERS OF CONNECTIVE TISSUE IN DUPUYTREN'S CONTRACTURE: RELATION TO POSTOPERATIVE OUTCOME

Stephan Wilbrand,[1] Christina Flodmark[1] Anders Ekbom[2] and Bengt Gerdin[3]

From the Departments of [1]Hand Surgery, University Hospital, Uppsala, [2]Medical Epidemiology, Karolinska Institute, Stockholm, [3]Plastic Surgery, University Hospital, Uppsala, Sweden

Abstract. We investigated 103 consecutive patients operated on for Dupuytren's contracture (DC) to find out the relation between the expression of activation markers of connective tissue in surgical specimens obtained prospectively and recurrence of disease. The history of the disease and present state of the operated hand were obtained a mean of 4 years (range 2.5–6) after the latest operation. Immunohistochemical staining for anticollagen type IV, integrin α_5, laminin, smooth muscle α-actin, procollagen type I, and desmin was evaluated. Almost half of the patients noticed recurrences during the study period, one fifth within six months of operation. No differences in the expression of any of the markers investigated were found, either earlier or later than six months postoperatively, in patients with or without recurrent bending. Furthermore, there were no associations between sex, age at onset, number of operations, heredity, diabetes mellitus, or drugs taken for cardiovascular disease, and the expression of any of the immunohistochemical markers. The individual characteristics that place a person at high risk are not obviously related to ongoing production of connective tissue at the time of operation or to connective tissue activity in its conventionally-used sense.

Key words: Dupuytren's contracture, immunohistochemistry, recurrence, connective tissue, collagen.

Correspondence to: Stephan Wilbrand, MD, PhD, Department of Surgery, Enköping Hospital, SE 745 25, Enköping, Sweden. (Tel: +46-171-418268. Fax: +46-171-440769. E-mail: stephan.wilbrand@plastik.uu.se)

Recurrence after operation for Dupuytren's contracture (DC) is common and remains an obvious problem for affected patients. Widely varying recurrence rates (0 to 78%) have been reported, depending on the selection of patients and procedures among other factors (8, 10, 11, 26). Recurrence rates depend on length of follow-up and on the type of procedure done. Recurrence is more common among patients with early onset (younger than 40 years), a family history of the disease, bilateral disease, ectopic lesions, and in those with "Dupuytren's diathesis" (13). Each recurrence and each reoperation give an increased risk for complications (2, 29).

The most potent known aetiological factor in DC is heredity, but the exact aetiology is still unknown. Other risk factors that have been reported include smoking, alcohol misuse, diabetes mellitus, high cholesterol and triglyceride concentrations, AIDS, and epilepsy (9, 30, 31).

The condition is characterised by an abnormal proliferation of fibroblasts and their differentiation into myofibroblasts (33), and changes in the expression of extracellular matrix (ECM) proteins (17), which result in fixation of the skin and flexion of the affected fingers. The major biochemical features of DC are an increase in total collagen (36) and an increase in the relative content of type III collagen (3, 22) in proportion to the extent of involvement of the aponeurotic tissue (20). There are also altered characteristics of collagen with an overhydroxylation of lysyl residues (27) and decreased cross-linking (21). Finally, there is an increase in proteoglycans compared with normal palmar fascia (6), as well as increased expression of integrin α_5 and its extracellular ligand, fibronectin (16).

Earlier studies have shown that the disease has three histological stages: proliferative, involutional, and residual (15). While the proliferative stage has been related to an active phase of the disease, it has been suggested that the involutional phase indicates the progression of contraction (19), and the residual phase the end stage.

The present study was based on the concept that these three stages are steps in a natural disease process. We therefore hypothesised that operating on patients in

a presumed "end stage" of the process, the residual stage, would be associated with lesser risk of recurrence than in patients operated on during the earlier phases. As an extension of this we also hypothesised that characteristics of connective tissue that reflect various aspects of biological activity would be related to the risk of recurrence. These hypotheses were evaluated in a prospectively studied group followed up more than two years postoperatively. Biopsy specimens taken at operation were investigated using immunohistochemistry to study various markers that reflect activation of connective tissue.

PATIENTS AND METHODS

Patients and surgical details

One hundred and three consecutive patients were investigated who had local fasciectomy for Dupuytren's contracture between 1994 and 1998. The indication for operation was a digital contracture of more than 40°. Informed written consent was obtained from all patients. The entire surgical specimens were retrieved for analysis.

The study was approved by our institutional review board on the use of human subjects in research.

Patients' assessment of recurrence

A questionnaire was sent out to patients a mean of 4.4 years after operation (median 4.5; range 2.5–6) (see Appendix). We defined the term "recurrence" based on the patient's response to "the fingers operated on are beginning to bend again" in one of the questions, or from reports of medical examinations in the patient records.

Handling of biopsy specimens and immunohistochemical staining

Tissue from the surgical specimens was preferentially sampled from nodular areas, as myofibroblasts proliferate almost solely in these areas (35). The specimens were trimmed and snap frozen in dry-ice isopentane and stored at −70°C. Between one and five specimens (mean 3) were obtained from each patient. All specimens were first cryosectioned, stained with haematoxylin and eosin, and reviewed with respect to activity of DC. For each patient the specimen that contained the most active nodule was chosen for further evaluation after serial sectioning.

Serial cryosections were fixed in ice-cold acetone, rinsed in phosphate buffered saline (PBS), and incubated for 30 minutes with the primary antibody diluted in PBS and supplemented with 1% bovine serum albumin and 5% appropriate normal serum. After repeated rinsing, endogenous peroxidase was extinguished with 3% hydrogen peroxide in 100% methanol for 15 minutes. After incubation with biotinylated horseantimouse, or rabbitantigoat secondary antibody, they were stained with the avidin-biotin complex technique using Vectastain ABC-Elit kit (Vector Laboratories, Burlingame, CA, USA) with ethylcarbazole as the peroxidase substrate. Finally, the sections were counterstained with Mayer's haematoxylin. Goat *anticollagen type IV* was obtained from Southern Biotechnology Inc. (Birmingham, AL, USA). Mouse *anti-integrin* α_5, clone PID6, came from Chemicon International Inc. (Temecula, CA, USA). The Mab 3E5 (7), which recognises *laminin* and is used as a marker for basement membranes, was a gift. The anti-*smooth muscle α-actin* antibody, clone 1A4, was obtained from Sigma (St. Louis, MO, USA). Anti-*desmin*, which recognises an intermediate filament observed in most smooth muscle cells (5, 28), but also in some pericytes (24), was bought from Dakopatts (Glostrup, Denmark). Mouse anti-*human procollagen type I* came from Biogenesis Ltd (Poole, UK). It recognises the carboxyterminal part of procollagen type I, but not mature collagen fibres (18). Biotinylated horse-anti-mouse IgG came from Vector Laboratories (Burlingame, CA, USA). All antibodies were diluted in PBS and used in optimal concentrations after serial dilution. Mouse IgG or, when applicable goat IgG, was used as a negative control.

Microscopic evaluation

Haematoxylin-stained sections were staged according to Luck's classification (15) based on the degree of cellularity and fibrosis, and were differentiated into three phases: proliferative, involutional, and residual. Nodules in the proliferative phase are characterised by high stromal cellular density and pronounced vascularisation, while the ECM contains few mature collagen fibres. Nodules in the involutional phase also show high stromal cellularity, but the stromal cells tend to be aligned in the same direction. In the residual phase the nodules are hypocellular and the fewer aligned stromal cells are surrounded by thick fibres of collagen with a tendon-like appearance.

Immunohistochemical staining for anticollagen type IV, integrin α_5, laminin, smooth muscle α-actin, procollagen type I, and desmin was evaluated without knowledge of the history in a conventional light microscope at ×100 magnification. Slides were judged by two independent viewers and staining intensity within nodular tissue was assessed. Intensity and extent of staining were assessed and graded using a 0 to 3 ordinal scale: 0 = absent; 1 = minimal; 2 = moderate; and 3 = maximum. The prevailing intensity was taken to reflect the entire section.

Table I. *Distribution of hands and fingers operated on among the 93 evaluated patients*

Hand		Finger				
		Thumb	Index	Middle	Ring	Little
Right	45	2	2	6	29	33
Left	48	7	7	14	30	36

Statistical procedure

Relations between dichotomous variables were evaluated with the chi square test and Fisher's exact test, as appropriate. The predictive value of clinical and immunohistochemical variables on recurrence was calculated by logistic regression. In patients who had lesions biopsied on two or more occasions, relations between clinical variables and immunohistochemical findings were calculated for the first operation only.

RESULTS

Information was obtained from 93 patients (86 men and 7 women) who were alive at the time of follow-up, and for three of these the information was obtained from medical records and relatives. At the time of operation the patients' ages ranged from 25 to 86 years (mean 61; mean for men 60; mean for women 68). Forty-five right hands and 48 left hands were operated on (Table I). During the study period two thirds of the patients were operated on for primary disease and one third for recurrent disease. The surgical histories of all 93 patients are given in Table II.

The medical history and postoperative features are given in Table III. The mean age at the time of operation for the 18 patients who developed early recurrences was 57 years (range 26–84), which was

Table III. *History and postoperative features*

Feature	Number (%) of patients ($n = 93$)
Recurrent bending within study period	42* (47)
Within 6 months	18* (20)
Later than 6 months	24* (27)
Signs on soles of the feet	24 (26)
Family history	41 (44)
Father's side	27 (29)
Mother's side	14 (15)
Taking drugs for cardiovascular disease	40 (43)
Taking drugs for hyperlipidaemia	8 (9)
Diabetes mellitus	11 (12)
History of trauma	3 (3)
Taking drugs for epilepsy**	1 (1)
No reported illness or trauma	30 (32)
Smoking	
Current	11 (12)
"Ten years ago"	25 (27)

* $n = 90$; ** Extensive bilateral disease.

somewhat lower than for the study group as a whole (mean 61 years) (range 26–86). The mean age at operation for the 24 patients who reported recurrences during the study period was 58 years (range 26–84).

Of the 93 patients, 51 (55%) were classified as being in the residual phase, 28 (30%) in the involutional phase, and 14 (15%) in the proliferative phase. No differences were found in the distribution of histological phases in patients with recurrent bending of the finger or fingers operated on, either earlier or later than six months postoperatively, compared with patients without a history of bending postoperatively (Fig. 1).

Three or more previous operations predicted recurrent bending during the study period (logistic regression, $p = 0.03$; Fisher's exact test, $p = 0.04$), as did a family history of DC (logistic regression, $p = 0.04$).

Table II. *Surgical history*

| Feature	Number (%) of patients ($n = 93$)	Comment
Previous operations:	33 (35)	
Twice unilateral	6 (6)	
Three times	13 (14)	In 10 twice bilateral and once unilateral; in 3 three times unilateral
Four times	3 (3)	
Five times	3 (3)	
More than five times	8 (9)	
Operations during current study:		
Operation for primary disease; first operation	42 (45)	25 right hands and 17 left hands
Operation for primary disease; other hand previously operated on	18 (19)	
Operation for recurrent disease	33 (35)	
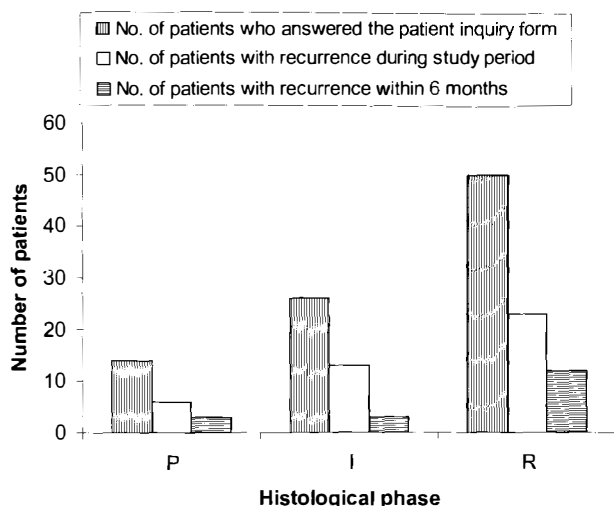
Fig. 1. Distribution of histological phases in the 90 patients who answered questions about relapse. P = proliferative phase; I = involutional phase; R = residual phase.

However, recurrence was not significantly related to the histological phase of disease, age at onset, or age at first operation. Early recurrence was, however, related to Dupuytren's diathesis defined from the additive value of "under 40 years of age at first operation", "presence of signs on the sole of the foot", and "family history of DC" (chi square = 0.01).

Immunohistochemistry showed that stromal cells of the highly cellular areas in both the proliferative and involutional phases were myofibroblasts, as illustrated by their cytoplasmic positivity for smooth-muscle α-actin, and also for desmin. In these areas there was also variable staining for procollagen type I. The ECM surrounding these cells strongly and diffusely stained for collagen type IV, laminin and integrin α_5, whereas no such immunoreactivity was found in the ECM of the fibrotic and hypocellular areas in the involutional and residual phases.

No differences were found in the expression of any of the markers investigated in patients with recurrent bending of the operated finger or fingers, either earlier or later than six months postoperatively, compared with patients without a history of bending postoperatively (Fig. 2). This was confirmed by a number of different statistical approaches including contingency table analysis with the chi square test and Fisher's exact test, and logistic regression where relapse was the dependent variable.

Furthermore, there were no relations between sex, age at onset of disease, number of operations, family history, diabetes mellitus, and drugs taken for cardiovascular disease on the one hand, and the intensity (grade) of the different immunohistochemical staining for collagen type IV, integrin α_5, laminin, smooth

muscle α-actin, procollagen type I, and desmin on the other.

DISCUSSION

The results of the present study indicate that the activity of connective tissue in the specimens assessed by immunohistochemistry was unable to indicate which patients were at high risk of recurrence, nor could we find any positive correlation between histological phase and higher risk for recurrence.

Patients with DC continue to trouble the clinician. Recurrence and extension of the disease are unsolved problems.

Widely varying recurrence rates (up to 78%) have been reported, depending on the length of follow-up and the selection of patients and procedures (10, 11, 26). Hueston found that most recurrences after operations for DC develop early (87% within two years) (12). Millesi, however, claimed that 48% of recurrences in his series occurred more than three years postoperatively (23). In 1985, Tubiana and Leclercq presented long-term results (mean follow-up of 10 years) (34). In their study recurrence was noted in 66% of the operated hands. Like Hueston, the authors emphasised the poorer prognosis in women, young patients, patients with ectopic lesions, associated disease, family history, and involvement of the little finger. Our figures, including 19% early recurrence, 45% recurrence during the study period, and 55% of the hands free of disease during the follow-up period, are somewhat lower than those of Tubiana and Leclercq, but the follow-up period in our study was shorter. The findings in Dupuytren's diathesis (12) – family history, presence of ectopic lesions, and early age at onset – are thought to influence the rate of recurrence. However, we found no over-representation of patients with recurrence and one single component of diathesis. The data do indicate that patients with more than one finding implicated in diathesis have a higher risk for early recurrence.

Skoog found a family history of DC in 22 of 50 cases. Eleven of 50 fathers, but only one mother, were affected (32). Ling obtained a family history of DC in only eight of 50 patients (14). The figure of 44% reported in the present study was somewhat higher. Although a family history is known to be the most potent aetiological factor for DC, our rate of recurrence is somewhat lower than reported in earlier studies.

The histological classification of the DC lesions into proliferative, involutional, and residual phases draws its inspiration from the concepts expressed by Luck and specified by Chiu and McFarlane (4). We found no significant relationship between the histological classification and the recurrence rate. This is actually in line
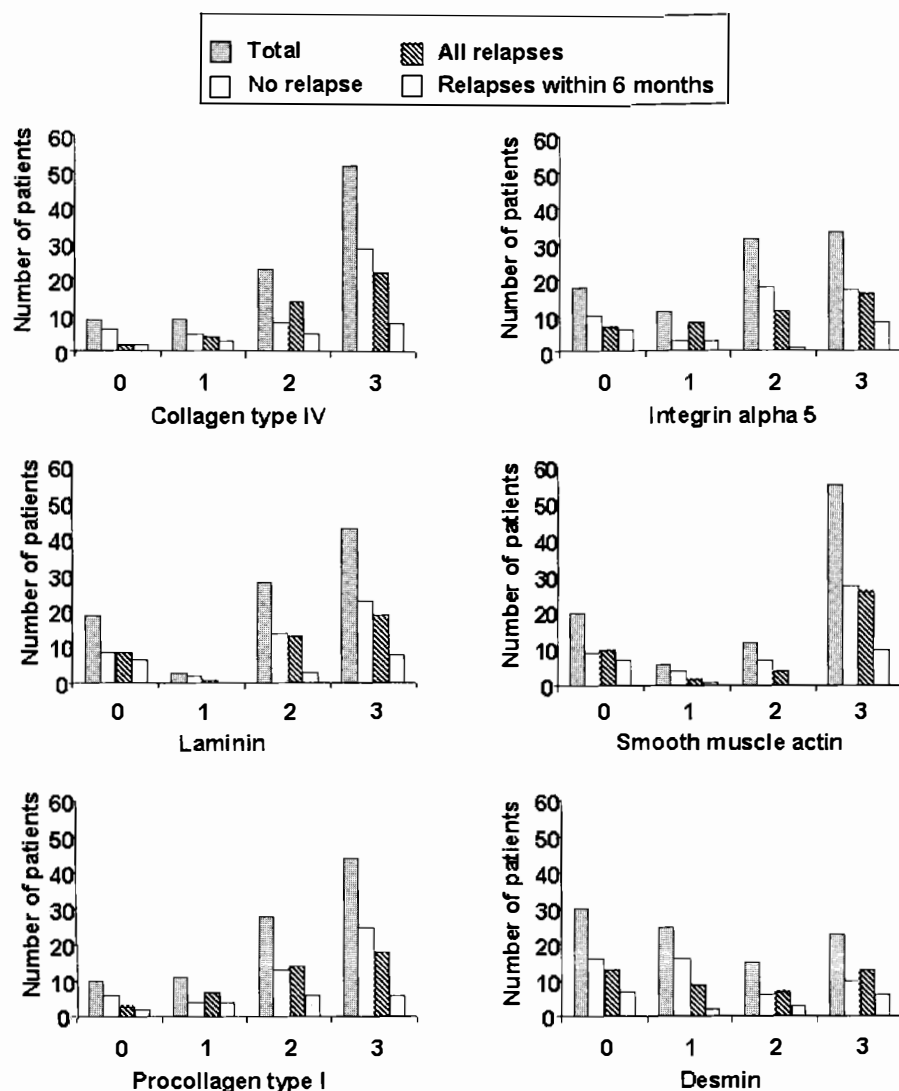
Fig. 2. Intensity of staining for different markers for connective tissue activation in all patients, those with relapse, and in those relapsing within six months after surgery. Intensity of staining is presented in four arbitrary steps where "0" reflects no staining and "3" indicates strong staining.

with the suggestions of Luck (15), Chiu and McFarlane (4), and Nézelof (25) that the three histological phases indicate three evolutional stages of the disease rather than reflecting something about the severity of the disease.

The chosen connective tissue markers were selected to identify differences in the expression of characteristics of myofibroblast phenotypes, that include desmin and smooth muscle α-actin, ongoing production of collagen type I, and a high fibronectin-binding ability of cells (1). These have all been related to an ongoing connective tissue proliferative process as such (33). The main finding of the present investigation is that those connective tissue markers are unable to identify individual patients who are at high risk of recurrence after operation for DC. It therefore seems that the characteristics that cause a person to be at high risk are not clearly related to ongoing connective tissue

production at the time of surgery or to connective tissue activity in its conventional sense.

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Appendix

Inquiry form for patients operated on for Dupuytren's contracture

Please mark the appropriate alternative or fill in your answers in the spaces indicated

1 Sex   ☐ Male   ☐ Female

2 Age   …………… years

3 How old were you (years) when your finger/fingers started to bend?

       Younger than 30   ☐

       30-40   ☐

       40-50   ☐

       50-60   ☐

       Older than 60   ☐

4 How old were you (years) when you were operated on for the first time for Dupuytren's contracture?

       Younger than 30   ☐

       30-40   ☐

       40-50   ☐

       50-60   ☐

       Older than 60   ☐

5 How many times have you been operated on for Dupuytren's contracture?

                                      <u>Left hand</u>   <u>Right hand</u>

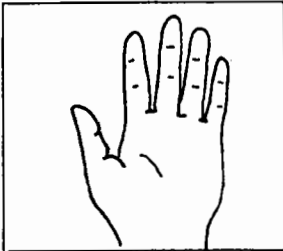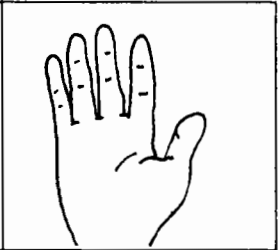   ☐ Never   ☐ Never

                                      ☐ Once   ☐ Once

                                      ☐ Twice   ☐ Twice

                                      ☐ Three times   ☐ Three times

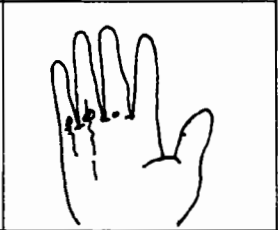   ☐ Four times   ☐ Four times
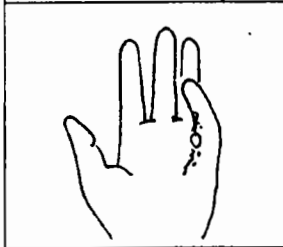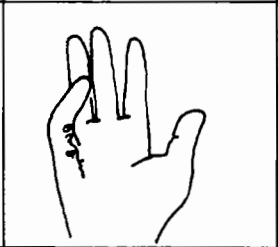
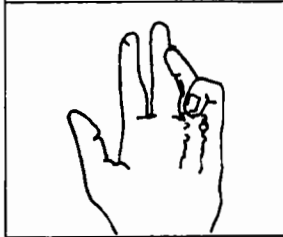   ☐ More than   ☐ More than
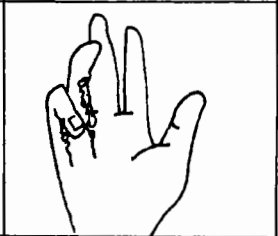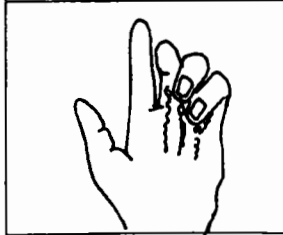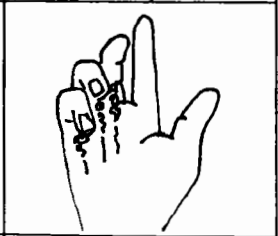
                                        four times   four times

6 Do you know of anyone in your family with   ☐ Yes   ☐ No

   Dupuytren's contacture?

   If "Yes", who? (mother, father, grandfather…)   ………………………………………...

7. How bent is/are your finger/s today? Put your hands on a table with the palms up. Look

carefully at both hands and compare them with the pictures below. Mark the picture that looks

like your hand.

	Left hand	Right hand
Normal hand		
A bump in the palm but no bent fingers		
One bent finger		
Two bent fingers		
More than two bent fingers		

8   Do you have a bump on your thumb ?   Left   Right

                                                        Yes   No   Yes   No

                                                         ☐   ☐   ☐   ☐

9   Do you have bumps on the soles of your feet?   ☐ Yes   ☐ No

10   Which statement reflects the status of your hand/

     hands? Mark the appropriate one.

       The latest hand operation improved my finger   ☐ Yes   ☐ No

       function

       The latest hand operation made my fingers   ☐ Yes   ☐ No

       straight

       My fingers are still straight   ☐ Yes   ☐ No

       The fingers operated on are beginning to bend   ☐ Yes   ☐ No

       again

       The fingers operated on bent within 6 months   .☐ Yes   ☐ No

       of the latest operation

       The fingers operated on always bend after   ☐ Yes   ☐ No

       every operation

       The latest hand operation made my fingers   ☐ Yes   ☐ No

       worse than they were before

       One or several fingers operated on are   ☐ Yes   ☐ No

       beginning to bend again

       I have problems using my hands   ☐ Yes   ☐ No

       I have problems using my hands for certain   ☐ Yes   ☐ No

       activities

11   Which daily activities do you have problems with

       because of your bent fingers? ....

       ....

12   Do you have pain in any finger when it is cold

       outside?

In one or more of the fingers operated on	☐	Yes	☐	No
In one or more of the fingers not operated on	☐	Yes	☐	No

13   Do you have diabetes?   ☐ Yes   ☐ No

14   Are you on regular drugs for some other illness?   ☐ Yes   ☐ No

       If yes, which ones? ....

15   Are you a smoker?   ☐ Yes   ☐ No

16   Were you a smoker 10 years ago?   ☐ Yes   ☐ No

       Do you have any comments about this inquiry

       form?

Thank you for your cooperation.