Endothelin — a novel regulatory peptide

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Endothelins are a family of structurally related vasoactive peptides synthesized by selected endothelial and epithelial cells. Endothelin is the most potent vasoactive biomolecule known to date. Recent evidences indicate that it is involved in several physiological and pathophysiological events. It will not be too long before the peptide stakes its claim to be the molecule of the decade.

SINCE the isolation of Endothelin-1 (ET-1) from porcine and human endothelial cells by Yanagisawa and his colleagues[1] and the subsequent identification[2] of the other members of the ET family, several studies have focused on the structure, expression and biological functions of these molecules. In a strikingly short span of three years, endothelins (ETs) have exploded upon our consciousness as biomolecules that are involved in smooth muscle contraction and vasoconstriction, the physiology and pathophysiology of cardiac, pulmonary and renal function, mitogenesis and tissue remodelling[3]. The story of ETs is all the more remarkable for the high degree of sequence homology they bear to the sarafotoxins isolated from the venom of the Israeli burrowing asp[4]. Apparently, genes encoding a snake venom toxin seem to have evolved into genes encoding an important mammalian regulatory peptide. Such are nature's whimsical ways!

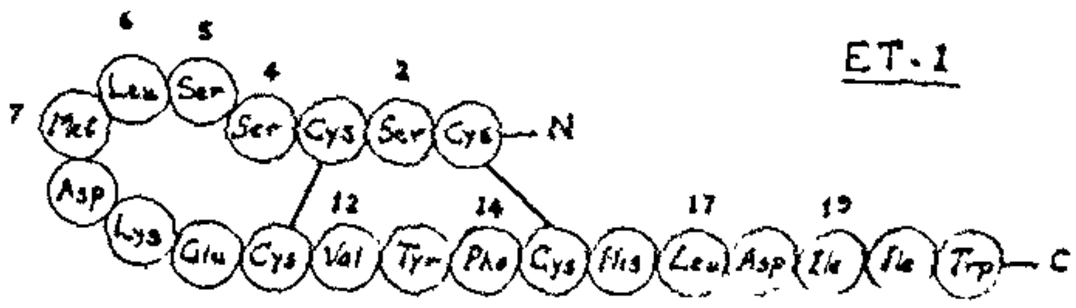
Biosynthesis

Endothelin is an acidic, 21-amino-acid peptide (≈ 2.5 kDa) made by the endothelial cells from preproendothelin (200 amino acids) and proendothelin (38 amino acids, called 'big ET') by proteolytic cleavage by an 'endothelin-converting enzyme'. Conversion of the precursor to the mature peptide is essential for bioactivity, and its prevention, therefore, has pharmacologic significance. Although originally thought to be exclusively an endothelial product, synthesis of endothelins has been identified in a number of tissues such as CNS neurons[5,6], avascular amnion[7] and renal[8] and respiratory epithelia[9,10]. Canine, porcine and human tracheobronchial epithelial cell cultures have also been shown to release these peptides[10,11].

Isopeptides, tissue-specific expression

Interestingly, ETs are found in three distinct isoforms—ET-1, ET-2 and ET-3—encoded by different genes[2] (Figure 1). A novel peptide, expressed predominantly in the intestine is termed vasoactive intestinal constrictor or endothelin-β and is reckoned to be a fourth isoform[12]. The isopeptides are differentially expressed in specific tissues[3]. For example, prepro ET-1, and not prepro ET-3, is expressed by cultured endothelial cells from large vessels and microvessels. mRNA for prepro ET-1 and ET-3 are abundant in foetal lung, spleen, pancreas and, to a lesser extent, in foetal kidney, atrium and ventricle. Transcripts for prepro ET-1 and ET-3 have been demonstrated in adult rat tissues as well. However, attempts to demonstrate ET gene expression in some adult tissues have not been very successful, probably due to the insensitivity of Northern blot analysis to detect the transcripts or poor hybridization of the ET-1 and ET-3 probes with noncomplementary tissue-specific isoforms. Nevertheless, it is clear that ET gene expression is regulated at both tissue-specific and developmental levels.

It is still not clear how ET gene expression is regulated. In endothelial cells, ET synthesis is reported to be stimulated by thrombin, transforming growth factor B, calcium ionosphores and epinephrine[13]. Nuclear factor-1, AP-1/JUN and other acute phase reactant regulatory elements have been identified in the 5'-flanking region of the human ET gene. The presence of these regulatory elements is consistent with demonstrations that TGF-B (possibly acting through nuclear factor-1), phorbol esters and IL-1 act to elevate prepro ET mRNA levels[13].



Amino acid number

Peptide	2	4	5	6	7	12	14	17	19
ET-1 (Human and porcine)	Ser	Ser	Ser	Leu	Met	Val	Phe	Leu	Ile
	Differences from ET-1								
ET-2 (Human)				Trp	Leu				
ET-3 (Rat)	Thr	Phe	Thr	Tyr	Lys		Tyr		

Figure 1. Structure of ET-1 and position of amino acid substitutions of ET-1 for ET-2 and ET-3. (From ref. 19)

Biological actions

ET receptors

The tightly regulated expression of the ET gene *in vivo* suggests a large array of cellular functions for these peptides. Further, ETs act in diverse cells and tissues, and receptor-mapping studies have demonstrated saturable, specific-binding sites for (^{125}I)-ET ($K_d \approx 0.5$ nM) in a number of foetal and adult organs, including the lung, kidney, heart, intestine, adrenal gland, eye and brain[14–18]. The density of binding sites is particularly high in the lung and heart[14,16,17]. Moreover, it has been shown that (^{125}I)-ET binding is not displaced by Ca^{2+} channel blockers, peptide neurotoxins, adrenergic agonists or vasoconstrictors, implying that ET binds to a specific cognate receptor and not directly to an ion channel or other non-specific receptors.

A striking feature of the biological action of ETs is that although they are of endothelial or epithelial origin, they act in a paracrine fashion on nearby smooth muscle or connective tissue cells to elicit an amazingly wide range of biological responses (Table 1). Importantly, they show vasoconstricting, bronchoconstricting and growth-promoting properties as they act on blood vessels and cardiac and non-cardiac tissues.

Table 1. Biological actions of ETs

Haemodynamic effects

(Regional differences in vasoconstriction exist)

Cardiac effects

Evoke positive inotropic and chronotropic effects on myocardium

Stimulate intense vasoconstriction of coronary arteries

Regulate muscle-specific gene expression

Neuroendocrine effects

Increase plasma levels of ANF, renin, aldosterone and catecholamines

Modulate synaptic transmission in hypothalamus and pituitary, act to release substance P.

Renal effects

Increase renal vascular resistance

Decrease glomerular filtration rate and renal blood flow

Increase Na^+ reabsorption through haemodynamic actions

Decrease Na^+ reabsorption through inhibition of Na^+, K^+-ATPase

Smooth muscle effects

Contract vascular and nonvascular smooth muscles

Mitogenic effects

Stimulate mitogenesis in vascular smooth muscle cells, 3T3 fibroblasts and glomerular mesangial cells

Gene expression

Influence expression of *c-fos*, *c-myc* and *VL30* genes

From ref. 3

ETs as potent vasoactive agents

The vascular endothelium is ideally located to transduce chemical signals within the blood stream and to respond to changes in intraluminal pressure and flow. ET-1, one of its products, is found to be a more potent vasopressor agent than angiotensin II, vasopressin or epinephrine, its EC_{50} being approximately 1 nM. It has contractile action on a variety of blood vessels from a number of species including the rat, rabbit, dog and human, the effect being more pronounced on veins than on arteries[19]. Further, available data also indicate that the haemodynamic response to these peptides is rather complex, depending as it does on the vascular bed under study, the dose, route and rate of administration of the peptide as also on the initial degree of vasomotor tone[19].

Interestingly, the endothelium also elaborates substances that have a vasorelaxing effect[20]. The modulatory role of endothelium-derived relaxant substances on the vasoconstrictive effects of ET could not be recognized in the initial study of Yanagisawa, who examined the contractile response to ET using arterial segments with a damaged endothelial cell layer. (Providence can be unkind, at times!) Subsequent studies have shown that the half-life of ET in the blood stream is approximately 1–2 min but before its clearance from the blood, it induces the vascular endothelial cells to release vasorelaxing factors such as endothelium-derived relaxing factor, atrial natriuretic peptide and prostacyclin, thereby limiting its own vasoconstricting effects[20].

Admittedly, the endothelial cells lining the blood vessels would not secrete substances so potently active on vascular smooth muscle if not for some crucial physiological purpose. It would be fascinating to see how the ETs, along with other factors of endothelial origin, are involved in the impeccable regulation of vascular tone and blood pressure. The interplay of these potent vasoactive agents is so complex that one would wonder how 'a harp of a thousand strings can keep in tune so long'—and so well.

But, in the theatre of the endothelins, one does hear the odd note of discordance, and the dividing line between physiological purpose and pathological portent gets distinctly thinner.

ET—a boon or a bane?

The vasoconstricting effect of ET implies that it may play a role in hypertension. This is consistent with the

observation that renal arteries from spontaneously hypertensive rats are more sensitive to ET than those from normotensive rats[21]. Significantly, phosphoramidon, a neutral protease inhibitor, blocks the conversion of big ET to ET-1 and prevents the rise in blood pressure induced by injections of big ET in rats[22]. Phosphoramidon also lowers the blood pressure of spontaneously hypertensive rats[22].

It has been suggested[23] that ET may be involved in the pathogenesis of coronary spasm in patients with variant angina since the peptide can sensitize vascular segments and enhance vasoconstrictor responses. Specific antagonists of the ET receptor or inhibitors of the synthesis of the peptide should therefore be appropriate tools in the management of variant angina.

Several laboratories have reported significantly elevated levels of ET-1 in patients with essential hypertension, Raynaud's syndrome, vasospastic angina, acute myocardial infarction, renal failure, shock and toxaemia[24]. However, if ET acts only as an autocrine/paracrine local substance, ET in the circulation could be just the result of an 'overflow' of the peptide produced at sites of endothelial injury. The significance of plasma levels of ETs remains, by and large, unclear.

ETs and bronchoconstriction—a possible role in asthma

The contractile effect of ET is not limited to vascular smooth muscle. ET-1 contracts the stomach, duodenum, urinary bladder, bronchi and trachea. Enhancement of rhythmicity and magnitude of contraction in response to ET-1 in isolated rat uterine horns have also been reported. Recent evidences point to a possible role for ET in the causation of bronchoconstriction associated with asthma[25]. Of particular significance is the demonstration of ET-like immunoreactivity in the bronchoalveolar lavage fluid, which would suggest that ET exists in the human respiratory tract[26]. Identification of receptors for the peptide on bronchial smooth muscle of rats, pigs and man by autoradiography and isolated membrane-binding assays are consistent with the known contractile action of ET on smooth muscle[25]. Further, the bronchoconstrictor effect of inhaled ET-1 in guinea pigs[27] and the elevated levels of ET-1 in the serum in acute asthma[25] show that ET may be involved in production of brochoconstriction that characterizes asthma. The stimulation of 15-lipooxygenase activity by ET-1[28] and the consequent generation of 15-hydroxyeicosatetraenoic acid, a chemoattractant for leucocytes, including eosinophils, could contribute to airway inflammation. Further, the proliferative effect of ET-1 on fibroblasts may explain, in part, the airway collagen layer thickness in asthma.

ETs and regulation of gene expression

Mitogenic action

In addition to the transient effects on smooth muscle contraction discussed above, the peptides are known to regulate gene expression to produce long-term effects. ET is found to be a potent mitogen for cultured cells and it has been shown to induce expression of the *c-fos* gene in glomerular mesangial cells[29], vascular smooth muscle cells[30] and 3T3 fibroblasts[31]. Inositol lipid turnover and protein kinase C activation represent one of the important mechanisms by which the peptide exerts its mitogenic effects[31]. ET has recently been shown to stimulate tyrosine phosphorylation of a number of substrates and this may occur via protein kinase C dependent and independent pathways[32]. Tyrosine phosphorylation of cellular proteins is a critical component of the mitogenic response to various growth factors and the effect of ET on tyrosine phosphorylation could be relevant to its mitogenic effects.

c-fos gene expression

Expression of the *c-fos* gene has been reported to occur rapidly upon addition of ET-1 to various cell types[3]. ET is a more potent inducer of *c-fos* gene expression in mesangial cells than 5% faetal bovine serum. Expression of the gene is maximal at 30–60 min and is undetectable by 120 min, which is characteristic of *c-fos* expression by several agonists. *c-fos* induction is achieved by multiple independent signal transduction pathways, such as the one involving the serum response element (SRE) located 300 bp upstream of the transcription start site[33], the cAMP response element (CRE)[34] or via increments in $[Ca]_i$ (ref. 3). It appears unlikely, at this point of time, that ET increases *c-fos* transcription by the CRE-sensitive pathway. Available evidence points to the possibility that by activating the phosphoionositide cascade, ET enhances both protein kinase C activity and $[Ca]_i$. As induction of *c-fos* via SRE is already documented, it is possible that ET activates *c-fos transcription* via the SRE or Ca^{2+}-sensitive pathways[3]. Moreover, ET also increases transcription of the *c-myc* (ref. 30) and VL30 (ref. 35) genes. Activation of VL30 genes is particularly interesting as these are activated by other promitogenic agents.

ET and cardiac hypertrophy

Recent evidences point to the possibility that ET may regulate cardiac growth and development. The identification of high-affinity ET-1 receptors in the ventricular

myocyte[36] is consistent with the suspected role of ET in the regulation of cardiac growth through hypertrophy of existing myocardial cells. It has been shown that continuous stimulation by ET-1 can lead to activation of muscle-specific genes in terminally differentiated cardiac cells[37]. ET-stimulation of neonatal rat myocardial cells results in an increase in the steady-state levels of mRNA encoding an individual contractile protein and leads to reactivation of an embryonic gene (ANF) in ventricular myocytes[37]. Studies employing transient expression of MLC-2 and ANF luciferase fusion genes demonstrate the activation of these two muscle-specific promoters, thereby indicating that ET-1 generates signals which ultimately reach the nucleus and orchestrate the transcription of these two cardiac muscle genes[37].

In vivo, cardiac hypertrophy is associated with the activation of immediate early gene expression[38], the activation of contractile protein gene expression[39,40] and the reactivation of a programme of embryonic gene expression[38,40]. ET-1 appears to be inducing all these changes *in vitro*. Further, stimulation of neonatal rat myocardial cells by ET-1 leads to morphologic, structural, biochemical and genetic alterations, which are indistinguishable from those induced by α-adrenergic agonists, suggesting that ET-1 might indeed be a bonafide hypertrophic stimulus for adult myocardial cells[37].

Earlier studies had assumed that myocardial cell hypertrophy is the result of a direct effect of hormones and mechanical stretch on myocardial cells. The role of neighbouring nonmuscle cells in the control of myocardial growth has not been studied adequately. Since ET-1 is released from endothelial cells, which lie immediately adjacent to the myocytes within the intact myocardium, the activation of myocardial hypertrophy by ET-1 represents a potentially important paracrine mechanism for the regulation of myocardial growth and development. Regulation of gene expression by ET can thus account for complex events, leading up to mitogenesis, and vascular and tissue remodelling in disease. The question whether ET is a boon or a bane remains unresolved at this point of time.

Mechanism of action

Transmembrane signalling

Most contractile processes require an increase in $[Ca]_i$ to regulate actin–myosin interactions and generate tension. An increase in $[Ca]_i$ is undoubtedly one of the signals mediating the contractile action of ET on vascular and nonvascular smooth muscle and its inotropic action on cardiac atria. An appraisal of the effects of ET on Ca^{2+}-signalling and transmembrane signal transduction through the phosphoionositide cascade would therefore be to understand the short- and long-term effects of these regulatory peptides.

Ca^{2+} signalling

Ligand-receptor activated rise in $[Ca]_i$ occurs through increased net influx of Ca^{2+} across the plasma membrane, release of Ca^{2+} from intracellular stores or a combination of both mechanisms. As pointed out by Simonson and Dunn[3], three lines of evidence suggest that both mechanisms contribute to ET-Ca^{2+} signalling. First, ET-induced $[Ca]_i$ transients are attenuated and the sustained increase is abolished in Ca^{2+}-free medium. Second, pre-treatment with Ca^{2+}-chelators before addition of ET inhibits the spike increase and blocks the sustained phase. Third, when mesangial cells are loaded with BAPTA, the intracellular Ca^{2+} chelator, and incubated in Ca^{2+}-free medium, ET fails to increase $[Ca]_i$. It appears that both extracellular influx of Ca^{2+} and intracellular release contribute to the transient phase, whereas extracellular influx is primarily responsible for the sustained phase.

Ca^{2+} influx into cells is regulated by two types of Ca^{2+} channels—the voltage-operated channel (VOC) and the receptor-operated channel (ROC). Evidence exists that ET promotes Ca^{2+} influx via a dihydropyridine-sensitive Ca^{2+} channel (VOC). It has been reported that dihydropyridine channel blockers inhibit ET-induced contraction[41,42] as well as increments in $[Ca]_i$ (ref. 41) and ^{45}Ca uptake[42] in vascular smooth muscle cells, implying that ET activates VOC. Direct measurement of Ca^{2+} conductance by patch-clamp technique is again consistent with activation of VOC by ET[43]. Several lines of evidence also suggest that ET gates ROC in some systems where contraction in response to ET is unaffected or only minimally inhibited by dihydropyridine or phenylalkylamine channel blockers[44]. Further, ET-induced increments in $[Ca]_i$ are insensitive to blockade by dihydropyridine channel blockers even when the cells express readily activated VOC[45], indicating Ca^{2+} entry through ROC. Recent studies involving direct measurement of Ca^{2+} conductance by patch clamping reveal that ET promotes multiple pathways of Ca^{2+} entry in a single cell.

Phosphoinositide cascade

Release of Ca^{2+} from intracellular stores by ET is effected by a transmembrane signal transduction process involving the ET receptor at the cell surface, a coupling G protein and phospholipase C. It has been shown that ET stimulates a dose-dependent increase in inositol lipid turnover in vascular smooth muscle

cells[35,46,47], fibroblasts[31,35], atrial cells[48], and glomerular mesangial cells[29,49]. In target cells, phospholipase C hydrolyses phosphatidyl inositol 4,5-bisphosphate to form two second messengers, IP_3 and diacylglycerol[50]. IP_3 is water-soluble and hence diffuses into the cytoplasm, releasing Ca^{2+} from intracellular stores and elevating $[Ca]_i$. The neutral diacyl glycerol remains within the plasma membrane and activates protein kinase C which could contribute to ET-induced biological responses. The precise role of protein kinase C in ET-induced transmembrane signalling has not been clearly delineated. Nevertheless, protein kinase C appears to inhibit ET-induced Ca^{2+} signalling, thereby acting as a negative feedback signal[3]. Importantly, protein kinase C has been implicated in the promitogenic effect of ET[29,49].

It is possible that the ET-induced increase in $[Ca]_i$ and protein kinase C activity promotes the transcription of the *c-fos* gene whose product is known to activate transcription of gene networks. It has been proposed by Simonson and Dunn[3] that, through formation of leucine zippers, newly synthesized c-fos protein forms heterodimers with other related *trans*-acting factors. These heterodimers in turn bind to *cis*-acting sequences on target genes to amplify short-term responses into long-term changes in gene expression. A tentative model for cellular signalling by ET, proposed by Simonson and Dunn[3], is shown in Figure 2. It would appear that a lot has been done but, with endothelins, surely, the best is yet to be.

Concluding remarks

The discovery of endothelin was perhaps the inevitable outcome of the curiosity of a graduate student, Masashi Yanagisawa, who felt impelled to inquire into an interesting report from the laboratory of Highsmith and his colleagues[51] that endothelial cells in culture elaborate into their medium a potent vasoconstrictor substance. As the story of the endothelins unfolded rapidly, several groups of investigators set out to study the biological effects of the peptides. The results of their investigations, briefly outlined in this review, reveal that endothelin is not only a remarkable molecule in itself; its manner of expressing its 'dictates'—as indeed they are—is, at the same time, ingenious, direct, subtle and certain. (In contemporary scientific parlance, one would perhaps say—autocrine, paracrine, intracrine!) Any attempt at elucidating the mechanism of action of endothelins is bound to prove rewarding for it would contribute to a better understanding of the physiological and pathophysiological functions of a novel regulatory peptide 'that has come home to roost'[22].

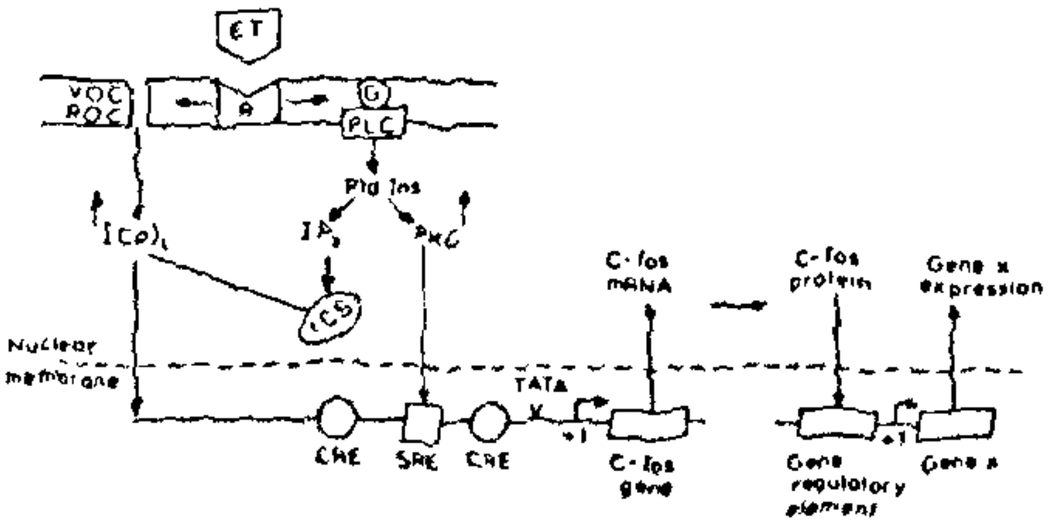


Figure 2. A model for cellular signalling by ET. (based on ref. 3) (ET, endothelin; R, receptor; G, G-protein; PLC, phospholipase C; PtdIns, Phosphatidyl inositol; VOC, voltage-operated channel; ROC, receptor-operated channel, IP_3, inositol (1,4,5,) triphosphate; PKC, protein kinase C; CRE, cAMP response element, SRE, serum response element; ICS, intracellular Ca^{2+} store)

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RESEARCH ARTICLE

Analysis of landslide sites: Kilbury Road, Kumaun Himalaya, India

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Landslides occur at sites with steeper hillslopes, fewer trees, and rocks with more horizontal but fewer vertical discontinuities. Environmental controls are better indicators of landslide incidence than landslide size. Size is a function of the history and status of the landslide system. Rockfall-type instabilities are associated with frost activity on colder slopes, slump-type instabilities with high apparent dip of rocks, greater depths of beneath-soil regolith, and warmer, wetter, sites. Correlations with slope-aspect indicators seem independent of geological control. We have measured 153 landslides, average size 18 cubic metres, along a 7.4 km stretch of roadway, and report our findings here.

LANDSLIDE debris is a major problem for the Himalayan road network[1]. Each monsoon triggers instabilities capable of dumping perhaps 550 cubic metres of debris on each kilometre of road-bed[2]. This paper is a case study of 153 landslides measured along a 7.4 km stretch of Himalayan highway: the Kilbury Road, Nainital, More than 75% of this road way receives landslide debris.

Most of the landslides are composite features with a complex development extending over several years. Little meaningful classification is possible except to distinguish a continuum that stretches from 'rockfalls' (TYPE = 1-landslides), where debris moves as a discrete block under the control of gravitational processes, to 'slumps' (TYPE = 3), where movement involves water and an element of rotation/deformation, through a range of hybrid and intermediate features (TYPE = 2)[3,4]. A survey of average conditions between each 200-m benchmark along the road indicated that 39% of the roadcut was affected by rockfall and 38% by slumping[5].

The Kilbury Road, Nainital (29° 24′ N, 79° 28′ E), was built in the eighties. It runs, at an altitude of around 2200 m, across the upper convexity of a steep, northeast-facing, hillside through relatively undisturbed forest. It is cut through sedimentary and low-grade metamorphic rocks, mainly slates (Blaini–Infra–Krol and Krol formations)[6]. The rocks are massively folded and faulted but on average they dip westwards at about 34°. The road is 6.2 m wide (σ 1.3) and backed by a roadcut which is 7.7 m high (σ 3.8) and inclined at an angle of 74° (σ 6) (Figure 1). The survey covers 7.4 km from Tanki to a little beyond Kilbury.

Data collection

This is part of a long-term study. Previous publications examined correlations between the percentage of each 200-stretch of road-bed affected by different types of landsliding and other factors in the environment[5,7]. In this article we examine a new, much larger, data set recorded at each significant landslide system. A landslide is called 'significant' if its outfall